MASTER THESIS IN DRUG ANALYSIS FOR THE DEGREE MASTER OF PHARMACY

ELECTROMEMBRANE EXTRACTION UNDER DIFFERENT IONIC STRENGTH CONDITIONS

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LIST OF ABBREVIATIONS

LLE	Liquid-liquid extraction
SPE	Solide-phase extraction
EME	Electromembrane extraction
SPME	Solide- phase microextraction
LPME	Liquid-phase microextraction
SDME	Single-drop microextraction
DLLME	Dispersive liquid-liquid extraction
HF-LPME	Hollow fiber liquid-phase microextraction
HCl	Hydrochloride
KCl	Potassium chloride
HCOOH	Formic acid
NaOH	Sodium hydroxide
NPOE	2-nitrophenyl octyl ether
ENB	1-ethyl-2-nitrobenzene
NPPE	2-nitrophenylphenyl ether
IPNB	1-isopropyl-4-nitrobenzene
DEHP	di(2-ethylhexyl) phosphate
TEHP	tris(2-ethylhexyl) phosphate
TTAB	tetradecyltrimethylammonium bromide
CE	Capillary electrophoresis
EOF	Electroosmotic flow
HPLC	High performance liquid chromatography
UV	Ultraviolet

SLM	Supported liquid membrane
NaCl	Sodium chloride
NaH_2PO_4	Monosodium dihydrogen phosphate
PP	Polypropylene
RSD	Relative standard deviation
µm	micrometer
mm	millimeter
ml	milliliter
mM	millimolar
nm	nanometer
µg	microgram
µl	microliter
pg	picogram
i.d	internal diameter
Rpm	revolutions per minute
µA	microampere
kV	kilovolt
mA	milliampere
mbar	millibar
AU	Absorbance Unit

ABSTRACT

The present work has for the first time demonstrated electromembrane extraction (EME) of basic drugs of different polarity from aqueous samples containing concentrations of NaCl up to 10 % (w/v). The purpose of this work was to investigate whether the extraction recovery of aforementioned basic drugs would be affected by the presence of NaCl in aqueous samples. Twelve out of twenty four basic drugs of different polarity were successfully extracted with EME under different concentrations of NaCl. From acidified aqueous samples (donor solution) (500 µl), the drug analytes migrated across a supported liquid membrane (SLM) consisting of 1-ethyl-2-nitrobenzene immobilized in the pores of a hollow fiber, and into an aqueous acidified acceptor solution (15 µl) present inside the lumen of the hollow fiber by electrical potential difference at 25 V. Within 5 minutes of operation at 25 V, recoveries in the range 12 -76 % were obtained after EME of pethidine, nortriptyline, methadone, haloperidol, loperamide, hydroxyzine, papaverine, promethazine, clomipramine, verapamil, pyrilamine and reserpine. The extraction recoveries were not affected by different NaCl concentrations, confirmed by a T-test with 95 % confidence.

Various parameters like applied voltage, extraction time and the chemical composition of the SLM were briefly investigated to improve the extraction recovery of selected basic drugs in aqueous samples containing different concentrations of NaCl. In this study, comparison of silanized-, polypropylene- and glass vials as donor compartment was accomplished for the first time in order to collect more information about whether the extraction recoveries were affected by the nature of the donor compartment in EME.

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1 Introduction

1.1 Background

Sample preparation technique is highly required when performing analytical research with complex samples such as biological fluids (plasma, serum, whole blood and urine) where the substance of interest (analyte) is present in a very low concentration, typically in the range of pg/ml -µg/ml [1, 2]. In order to obtain reliable data material from drug- and bioanalysis for qualitative and quantitative purpose, a robust sample preparation technique is crucial. It demands 80 % of the total analysis time and must exhibit satisfactory performance with respect to high enrichment and selectivity [1, 2].

There exist a wide range of various sample preparation techniques, where traditional liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have been reported as the most popular and frequently used technique for sample preparation of biological fluids. The concepts behind these extraction techniques are based on partitioning of the analyte between two immiscible solvents (LLE) and between a solid phase and an aqueous solvent (SPE) [2].

However, LLE and SPE techniques are considered time consuming and involved large quantities of toxic and expensive organic solvents. Consequently, the need for more environmentally friendly solutions has resulted in major improvement of these traditional sample preparation techniques during the last decade. To reduce the usage of hazardous solvents in sample preparation, liquid-phase-microextraction (LPME) is introduced as a miniaturization of LLE [3]. In LPME, target analytes are extracted from an aqueous sample solution into a water immiscible organic solvent. It is further divided into single-drop microextraction (SDME), dispersive liquid-liquid extraction (DLLME) and hollow fiber microextraction (HF-LPME) [4].

The principle behind HF-LPME is based on passive diffusion of target analytes, through a thin layer of organic solvent immobilized within the pores of a porous hollow fibre (creating a supported liquid membrane abbreviated as SLM) , and into an acceptor solution inside the lumen of the hollow fiber [5, 6]. The driving force in HF-LPME is passive diffusion promoted by pH gradient sustained across the SLM. The pH condition should favour deionization of the target analytes in the donor solution, and ionization of the target analytes in the aqueous

acceptor solution [6]. However, passive diffusion requires relative long extraction times to reach steady-state, which is the major drawback of HF-LPME and other microextraction techniques [4].

In 2006, electromembrane extraction (EME) was introduced as a faster micro scale sample preparation technique compared to HF-LPME [7]. The extraction principle was based on electrokinectic migration of charged analytes from a sample solution (donor solution), through a SLM and further into an acceptor solution promoted by electrical potential difference in the system [7]. EME provided efficient isolation, enrichment and clean-up of target analytes from complicated biological samples resulting in high analyte recoveries within minutes [7]. EME has been applied for extractions of different hydrophobic basic drugs [6-10], hydrophilic basic drugs [10-12], acidic drugs [13] and peptides [14, 15]. EME with complicated biological samples like human plasma [16, 17], whole blood [18], urine [19], breast milk [19] and most recently oral fluids [20] has been reported.

1.2 Aim of the study

EME has already demonstrated its great value as a miniaturized sample preparation technique in drug- and bioanalysis witnessed by an increasing stream of publications. However, the fundamental knowledge about EME was still limited. In order to collect more scientific information about the performance of EME, the main objective of this work was to investigate whether the extraction recovery of basic drugs of different polarity will be affected by different ionic strength conditions as this has never been studied in detail before. Ionic strength described the concentration of ions in a solution, which was highly relevant in complex biological samples like urine. Human urine samples normally contained additional ions, proteins, salts and other endogenic substances [21]. Thus, knowledge about EME under different ionic strength conditions was attractive for future applications of EME of biological urine samples. If the basic drugs of different polarity were successfully extracted into the acceptor solution, then the major part of the task was comprised by optimizing the conditions of EME to obtain the highest recovery. Different parameters were investigated, and the results (extraction recoveries) were evaluated based on repeatability and reproducibility characterized by relative standard deviation.

2 Theory

2.1 Electromembrane extraction

Electromembrane extraction (EME) was introduced in 2006 as a novel micro scale sample preparation technique [7]. The concept behind EME was migration of charged analytes from a sample solution (donor solution), through a supported liquid membrane (SLM), and into an acceptor solution by utilizing electrical potential difference as the driving force [7]. EME offered a fast and simple way to obtain selective isolation and pre-concentration of charged analytes from biological matrices. The micro scaled setup of EME ensured usage of small amounts of organic solvent, typically in volumes of µl [7, 10, 14, 19, 22].

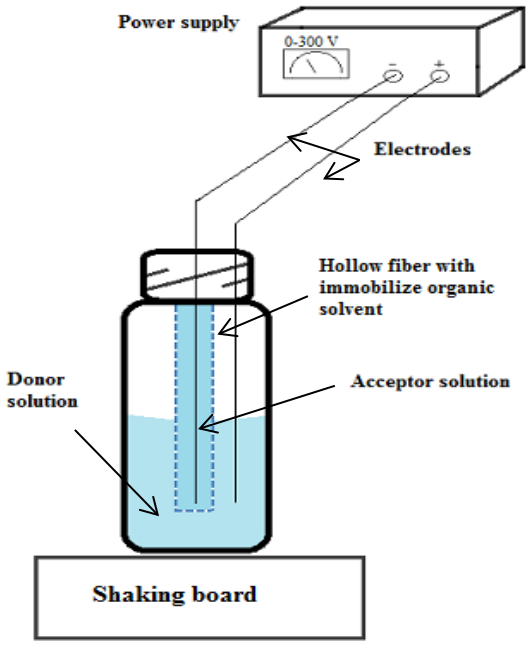

Figure 1 A schematic setup of EME

A schematic setup of EME is illustrated in Fig.1. The home-built extraction device consists of a vial filled with a donor solution, a porous hollow fiber filled with an acceptor solution and two platinum wires working as electrodes when connected to a power supply. The SLM is formed by immobilizing an organic solvent in the pores of the hollow fiber [7].

One of the electrodes is placed in the donor solution while the other is located in the acceptor solution inside the lumen of the hollow fiber. These electrodes are connected to a power supply, creating an electrical field as voltage is applied across the SLM. Consequently, charged analytes in the donor solution would migrate across the SLM towards the electrode of opposite charge in the acceptor solution effecting separation [7, 9]. Furthermore, ionization of the target analytes is required to enable electrokinectic migration. Thus, the pH in both donor and acceptor solution should be acidic for basic analytes, and alkaline for acidic analytes [7, 9]. During the experiments, sufficient stirring speed of the extraction device is crucial to induce convection of the analytes in the donor solution, thus reducing the stagnant boundary layer and maintaining a continuous motion of target analytes at the interface between donor solution and the SLM [7, 8]

2.1.1 Extraction kinetics across the supported liquid membrane

Nernst- Planck mass transfer equation are used to describe the steady-state mass transfer, J_j, for a charged analyte through the SLM in the presence of an electrical field (E) [9]:

$$J_j = -D_j \frac{dc_j}{dx} + \frac{D_j Z_j e E c_j}{kT} \tag{2-1}$$

where D_j is the ionic analyte diffusion coefficient, Z_j the charge of the analyte, c_j the concentration of the charged analyte in the SLM, x the distance from the SLM to the acceptor interface, k the Boltzmann's constant, e the elementary charge, and T represents the absolute temperature. The first part of Eq. (2-1) describes the diffusion of the charged analyte into the SLM, while the second part describes the electro migration of the charged analyte [6]. Eq. 2-1 is only valid for relatively thick membranes, for thinner membranes the equation is modified, since parts of the SLM are not electrical neutral [6]. A modified Eq. (2-2) is based upon the assumption that the analytes are mono charged and the SLM uncharged, resulting in a steady-state mass transfer of a given analyte (J_i) across the SLM can be calculated as [6]:

$$J_i = -\frac{D_i}{h}\left(1 + \frac{v}{ln\chi}\right)\left(\frac{\chi^{-1}}{\chi - \exp(-v)}\right)(C_i - C_{i0}\exp(-v)) \tag{2-2}$$

where D_i represents the diffusion coefficient for the ion, h the thickness of the membrane, C_i the charged analyte concentration at the SLM/donor interface, and C_{i0} the charged analyte concentration at the SLM/acceptor interface. v is defined as the dimensionless driving force in the system according to Eq. (2-3). χ is defined in Eq. (2-4), and describes the ratio of the total ionic concentration in the donor solution to that in the acceptor solution (ion balance) [4,6]:

$$v = \frac{z_i e \Delta\phi}{kT} \tag{2-3}$$

$$\chi = \frac{\sum_i c_{ih} + \sum_k c^*_{kh}}{\sum_i c_{i0} + \sum_k c^*_{k0}} \tag{2-4}$$

where z_i is the charge of the analyte, e the elementary charge, $\Delta\phi$ the electrical potential difference applied across the SLM, c^*_{kh} the concentration of the kth negative ion in the donor solution, c^*_{k0} the concentration of the kth negative ion in the acceptor solution, c_{ih} the concentration of the ith positive ion in the donor solution and c_{iO} the concentration of the ith positive ion in the acceptor solution.

Eq. (2-2) indicates that electrical potential difference, temperature, ion balance and the composition of the SLM are important parameters affecting the mass transfer of charged analytes across the SLM [6].

2.1.2 Ion balance

Theoretically, the mass transfer (J_i) across the SLM relies on the ion balance as shown in Eq. (2-2), and based on Eq. (2-4), a reduction of ion balance will increase the mass transfer. Since, the ion balance expresses the ratio between the total ionic concentrations on the donor side to that on the acceptor side, larger mass transfer of charged analytes can be expected if the ion concentration in the acceptor solution is high compared to the donor solution [6].

2.1.3 Supported liquid membrane and organic solvents

In EME, physical separation of the donor solution and acceptor solution is accomplished by utilizing a porous hollow fiber impregnated in organic solvent as the membrane [1]. The organic solvent is sustained in the pores of the hollow fiber by capillary forces, forming the SLM [3]. Target analytes pass through the pores of the hollow fiber from donor solution to acceptor solution creating a three phase system, thus adjustments to the SLM will affect the diffusion (D_i) and distribution of the target analyte in the membrane [6]. Optimization of a stable SLM is accordingly of great importance in order to achieve maximum extraction yields and selectivity within reasonable time, which in turn relies on the choice of organic solvent [7, 23].

When selecting a suitable organic solvent, there is some consideration to make regarding its properties. A suitable organic solvent should be water immiscible to prevent dissolution during the extractions, have a high boiling point to avoid evaporation and effectively immobilized in the walls of a porous hollow fiber to secure the quality of the physical barrier between donor and acceptor solution [8]. In addition to these properties, it is highly critical in EME that the solvent possesses a certain level of polarity which offered adequate electrical conductance to allow penetration of the electrical field [1]. The organic solvent should also ensure high donor-to-SLM distribution ratio for charged analytes. If the charged analytes have better solubility in the immobilized organic solvent than the donor solution, it will facilitate the migration across the SLM [12, 23].

Nitro-aromatic solvents as 2-nitrophenyl octyl ether (NPOE), 1-ethyl-2-nitrobenzene (ENB), 1-isopropyl-4-nitrobenzene (IPNB) and 2-nitrophenylpentyl ether (NPPE) are reported as efficient organic solvents for EME of basic drugs [4]. For acidic analytes, 1-octanol is reported as a more suitable SLM [1, 10]. Further investigation on the selectivity and chemistry of the SLM, has produced more knowledge about how to tailor-make the SLM for certain types of analytes [11]. For certain analytes of high polar character, the extraction efficiency is enhanced by addition of di(2-ethylhexyl) phosphate (DEHP) [11]. DEHP formed hydrophobic ion-pair complexes with the protonated polar analytes at the interface between the donor solution and the SLM, resulting in better distribution into the SLM [11].

2.1.4 Voltage

Based on Eq. (2-2) and Eq. (2-3), the mass transfer of charged analyte across the SLM is expected to increase with increasing voltage. This assumption is verified with experiments, and increased voltage facilitated higher mass transfer of analytes [6]. The current during EME is mainly relying on the voltage applied across the SLM. Thus, the entire extraction assembly including the donor solution, the SLM, the acceptor solution should behave like an electrical circuit in order to enable EME [1]. The electrical resistance of the system is to a large extent contributed by the SLM, and the organic solvent used as explained earlier must possess some level of conductivity to allow penetration of the electrical field [1]. Experience from experiments with current measurements also showed that the current in the system is influenced by a high initial concentration of charged analytes in the donor solution, and raised it to a relatively high level [6]. However, the current level should be kept as low as possible to prevent electrolysis and formation of bubble and gas due to the electrode reactions taking place during EME. Accordingly, generation of O_2 and H_2 occurs at the two electrodes located in the donor solution [7, 11, 15]:

Donor solution (anode): $H_2O \rightarrow 2H^+ + \frac{1}{2}O_2 + 2\,e^-$

Acceptor solution (cathode): $2H^+ + 2\,e^- \rightarrow H_2$

Bubble and gas formation is not a desirable situation during EME due to shifts in the pH conditions which reduce the extraction efficiency [12].

2.1.5 Extraction time

Papers have been published with experiments investigating recovery of different target analytes as function of EME time [6-8, 10, 13, 19]. Generally, recoveries increase with increasing extraction time up to a certain level, where the system enter steady-state conditions and no further gain in recovery is observed [1, 4, 13]. At the same time, experiments show that different analytes demand different extraction times to reach steady-state [4]. With mono charged analytes, increasing extraction time improve the recovery, whereas doubly charged analytes are not influenced by prolonged time of EME [4]. The explanation behind rapid extractions, where maximum recoveries are obtained after 5-10 minutes, is related to experiments performed in small donor compartments in the range of 300 µl - 2 ml [8]. Due to very short distance between the electrodes in small donor compartments, the impact of the electrical energy are stronger which intensify the mass transfer of analytes across the SLM hence improving the extraction efficiency resulting in shorter extraction time [7].

2.1.6 Extraction temperature

Based on Eq. (2-2) and Eq. (2-3), the temperature affects the mass transfer theoretically. A rise in the temperature decrease the dimensionless driving force of the system (v) and increase the diffusion coefficient (D_i) [6]. This assumption is verified by experimental data, the mass transfer of target analytes is highly temperature dependent [6]. However, the negative temperature influence on the dimensionless driving force of the system is insignificant against the positive effect temperature has on the diffusion coefficient [6]. The experimental findings conclude that the appropriate temperature for optimized extraction speed should be slightly above room temperature, except temperatures over 40°C which may lead to partial degradation of the SLM [6].

2.2 Capillary electrophoresis

Capillary electrophoresis (CE) is a well-established separation technique which is based on the transport of the electrically charged analytes in a solution under the application of an electrical field. Ions with different size and charge pass through the detector cell with different velocities. Positively charged ions migrate towards the negative electrode (cathode) because of electrostatic attraction, whereas negatively charged ions migrate towards the electrode of opposite charge (anode). Large ions demonstrate slower migration rates owing to higher resistance during electrophoresis compared to smaller ions. In addition to the size of the ion, the charge of the ion is vital to its electrophoretic mobility. Thus multiple charged ions travel faster in contrast to singly charged ions, a result of stronger attraction to the electrode of opposite charge. The principle of CE is illustrated in figure 2-2 [2].

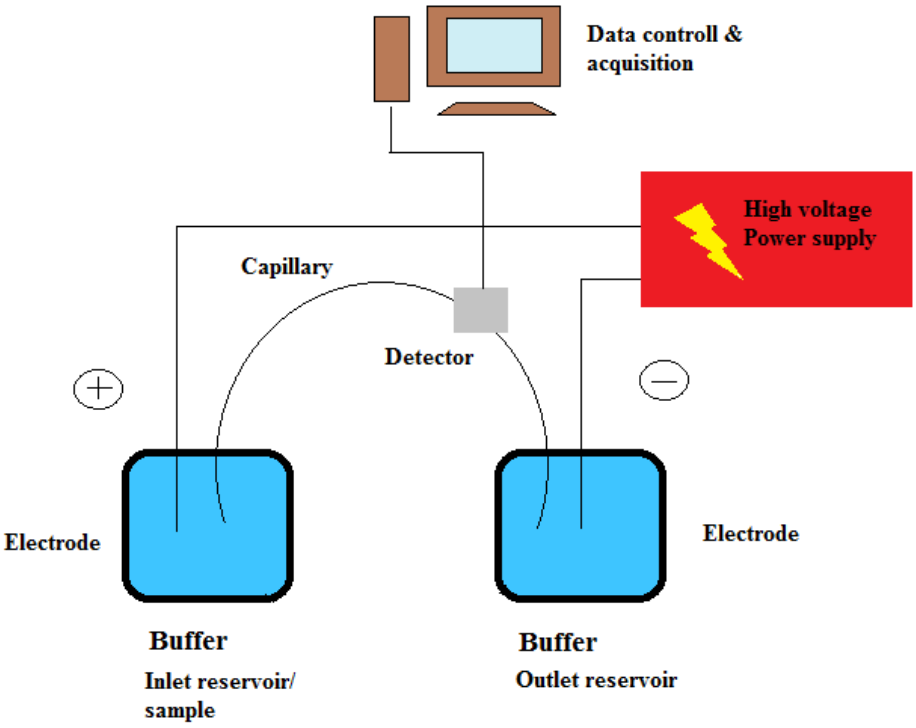

Figure 2 A General design of modern CE system. Analytes migrates from the positive potential in the inlet reservoir towards the negative potential in the outlet reservoir

The basic instrumental set up to accomplish CE consists of; buffer reservoirs with corresponding anode and cathode, a separation chamber (typically a fused-silica capillary), an injection system and an UV-detector. A capillary tube filled with buffer solution is placed between two buffer reservoirs. The electrodes are connected to a high voltage power supply which can often provide voltages up to 25-30 kV. Injection of the analytes is performed by replacing one buffer reservoir by the sample vial. A defined sample volume, typically in the nanoliter range, is introduced into the capillary by electrokinectic injection or hydrodynamic injection. An on-column UV-detector is located at the end of the capillary which is opposite to the injection site. A signal is created and plotted as a function of time when an analyte passes the detector cell and reduces the UV-light.

This plot is called electropherogram which showed a peak for each of the separated analytes during the CE analysis. Identification of the separated analyte relies on its migration time. Migration time for a given analyte is defined as the time the analyte passes the detector cell with maximum signal forced by applied voltage. The peak area is proportional with the concentration of the analyte in the sample. Thus quantitative and qualitative analysis can be performed in CE by measuring the migration time and the peak area of each analyte [15].

2.2.1 Indirect UV detection in CE

Identification and quantification with UV spectrophotometry, is based on the absorbance of the substance as function of wavelength. The absorbance of UV radiation requires an absorbing group of a molecule called chromophores. Double or triple bonds of organic compounds are chromophores and will contribute to the absorbance of UV radiation [2]. For experiments detecting inorganic Na^+ ions and Cl^- ions which lacked an UV- absorbing or fluorescence-absorbing chromophore, resulting in poor UV response, indirect UV-detection is employed. Indirect detection is achieved by using an absorbing ion in the buffer, which provide a high background absorbance [24]. When the absorbing ion is displaced by either the Na^+ ions or Cl^- ions in the sample, negative absorbance peaks is recorded in the electropherogram [24].

3 Experimental

Material and methods utilized in this work are listed under this section.

3.1 Drug analytes

Tab. 1 shows the physical-chemical properties of twenty four basic drug substances used as drug analytes in this work.

Table 1 Physical-chemical properties of the model drug analytes

Drug analytes	Molecular formula	Molecular weight	Log P	pK_a
Metaraminol	$C_9H_{13}NO_2$	167,2	-0,3	9,68
Cimetidine	$C_{10}H_{16}N_6S$	252,3	-0,1	2,3/4,5
Salbutamol	$C_{13}H_{21}NO_3$	239,1	0,7	9,4
Hydralazine	$C_8H_8N_4$	160,1	1,0	2,9/6,4
Mianserine	$C_{18}H_{20}N_2$	264,4	1,1	6,9
Pindolol	$C_{14}H_{20}N_2O_2$	248,3	1,5	9,7
Metoprolol	$C_{15}H_{25}NO_3$	267,4	1,6	9,7
Pethidine	$C_{15}H_{21}NO_3$	247,3	2,2	8,2
Procaine	$C_{13}H_{20}N_2O_2$	236,3	2,3	2,7/8,9
Hydroxyzine	$C_{21}H_{27}ClN_2O_2$	374,9	2,3	2,1/7,8
Pyrilamine	$C_{17}H_{23}N_3O$	285,4	2,7	5,3/8,8
Papaverine	$C_{20}H_{21}NO_4$	339,4	2,9	6,0
Haloperidol	$C_{21}H_{23}ClFNO_3$	375,8	3,8	8,1
Fluphenazine	$C_{22}H_{26}F_3N_3OS$	437,5	3,9	2,7/8,2
Perphenazine	$C_{21}H_{26}ClN_3OS$	404,0	3,9	2,7/8,2
Clozapine	$C_{18}H_{19}ClN_4$	326,8	3,9	3,9/7,4
Methadone	$C_{21}H_{27}NO$	309,5	3,9	9,1
Nortriptyline	$C_{19}H_{21}N$	263,4	4,0	10,5
Verapamil	$C_{27}H_{38}N_2O_4$	454,6	4,0	9,7
Loperamide	$C_{29}H_{33}ClN_2O_2$	477,0	4,1	9,4
Reserpine	$C_{33}H_{40}N_2O_9$	608,7	4,5	2,9/6,6
Prochlorperazine	$C_{20}H_{24}ClN_3S$	373,9	4,6	2,8/8,4
Promethazine	$C_{17}H_{20}N_2S$	284,4	4,9	9,1
Clomipramine	$C_{19}H_{23}ClN_2$	314,9	4,9	9,2

Log P obtained from https://scifinder.cas.org.
pK_a obtained from www.chemicalize.org

Twenty two of the basic drug analytes utilized in this work are obtained as pure material from Sigma–Aldrich (St.Louis, MO, USA), except from promethazine hydrochloride and procaine hydrochloride which are from Norsk Medisinaldepot (Oslo, Norway). List of other chemicals and solvents used in this work are further presented in Tab. 2.

Table 2 List of chemicals, quality and supplier

Chemicals	Quality	Supplier
Di(2-ethylhexyl)phosphate (DEHP)	**Min. 95 %**	**Sigma-Aldrich GmbH, Steinheim, Tyskland**
Monosodium dihydrogen phosphate-monohydrate ($NaH_2PO_4*H_2O$)	**Pro analysis**	**Merck KGaA, Darmstadt, Tyskland**
Sodium hydroxide (NaOH)	**Analytical reagent**	**VWR international**
2-Nitrophenyl octyl ether (NPOE)	**Selectophore**	**Sigma-Aldrich GmbH, Steinheim, Tyskland**
Orto-phosphoric acid (H_3PO_4)	**Pro analysis > 85 %**	**Merck KGaA, Darmstadt, Tyskland**
Tris(2-ethylhexyl)phosphate (TEHP)	**Unknown**	**Fluka Chemie GmbH, Buchs, Sveits**
Formic acid (HCOOH)	**Pro analysis 98-100 %**	**Merck KGaA, Darmstadt, Tyskland**
Ethanol (CH_3CH_3OH)	**96 %**	**Arcus (Oslo, Norway)**
Hydrochloric acid (HCl)	**Pro analysis 37 %**	**Merck (Darmstadt, Germany)**
Sodium hydrochloride (NaCl)	**Analytical reagent**	**VWR international**
1-Ethyl-2-nitrobenzene (ENB)	**Purum, ≥ 99 %**	**Sigma-Aldrich Chemie GmbH**
Acetonitrile (ACN)	**99,9 %**	**Merck (Darmstadt, Germany)**
Water (H_2O)	**Deionized, MilliQ**	**Millipore (Billerica, MA, USA)**

3.2 Equipment and procedures for electromembrane extraction (EME)

Fig. 1 in section 2.1 illustrates the basic setup of equipments used during EME. Based on earlier published work and knowledge, the experiments are accomplished by the following procedure: 500 µl of acidified donor solution is filled into a donor compartment (Agilent vials 32 x 11,6 mm, Matriks, Oslo, Norway). The upper end of a 7 cm piece of PP Q3/2 polypropylene hollow fiber (0,6 mm i.d, 200 µm wall thickness and 0,2 µm pore size, Membrana GmbH, Wuppertal,Tyskland) is open for introducing the acceptor solution, whereas the lower end is closed mechanically by a pair of pincers. The porous hollow fiber is dipped in an organic solvent (typically 2-etylnitrobenzene) for 5 seconds to form the SLM. Excess of solvent in the SLM is removed with a medical wipe. 15 µl of 10 mM HCl is filled

into the lumen of the hollow fiber with a micro syringe (Microliter syringes, Hamilton, Bonadus, Switzerland) and served as acceptor solution.

Finally the impregnated hollow fiber with the acceptor solution is placed into the donor compartment through a punched hole in the cap. Two platinum wires of 0,2 mm diameter (K.A. Rasmussen, Hamar, Norway) are used as electrodes and connected to a d.c. power supply with a voltage in the range of 0-300 V, and provided currents in the range of 0-450 mA (ES 0300-0.45 model, Delta Elektronika, Zierikzee, The Netherlands). The anode was then placed in the donor solution, whereas the cathode was placed in the acceptor solution. The extraction unit is agitated with a stirring rate of 900 rpm (Vibramax 100 agitator, Heidolph, Kelheim, Tyskland). After EME in room temperature (typically for 5 minutes), the acceptor solution is collected with a micro syringe and transferred directly to a micro insert for analysis in the CE-UV. In cases where the experiments are analysed in the HPLC-UV, 10 µl of the extracts are diluted with 30 µl mobile phase A.

3.3 Capillary electrophoresis

The CE instrument used for analysing was Agilent Capillary Electrophoresis System (Agilent Technologies, Santa Clara, CA, USA) coupled with a UV detector. Interpretation of the results was done on the Agilent ChemStation program (Agilent Technologies, Santa Clara, CA, USA). Separations were carried out in a fused-silica capillary with an internal diameter of 75 µm and an effective length of 55 cm (Polymicro Technologies, Phoenix, AZ, USA). Tab. 3 showed the conditions for the CE-UV instrument.

Table 3 CE-UV instrument conditions

Parameter	Value
Voltage	**30,0 kV**
Current	**50 – 100 mA**
Detection wavelength	**200 nm**
Injection	**hydrodynamic, 50,0 mbar in 5 seconds**
Separation buffer	**25 mM phosphate buffer pH 2,7**
Temperature in the cassette	**25 °C**

The first time the fused-silica capillary was taken into use, it had to be flushed with 100 mM sodium hydroxide for 30 minutes, with water for 5 minutes and finally with the separation buffer for 30 minutes. The daily rinsing procedure of the capillary was performed prior to the

analysis, and consisted of flushing with 100 mM NaOH for 15 minutes, water for 5 minutes and finally separation buffer for 15 minutes. The daily ending procedure was essential before shutting down the CE-UV instrument, and it was accomplished by flushing the capillary with water for 3 minutes and finally with air for 1 minute. The separation buffer was also cleared and replenished every 10^{th} analysis. Additional treatment of the capillary prior to each run was programmed flushing with 100 mM NaOH for 2 minutes and with separation buffer for 2 minutes.

3.4 HPLC-UV

A gradient elution program as seen in Tab. 4 was selected for the analysis in the HPLC-UV instrument (Agilent 1200-system, Agilent Technologies, Santa Clara, CA, USA) and the software ChemStation LC 3D was used for data interpretation.

Table 4 HPLC-UV instrument conditions

Parameter	Value
Column	**YMC- Triart C18 150 x 2,0 mm, I.D. 5 µm (YMC Europe, GmbH, Dinslakten, Tyskland)**
Mobile phase A	**95 % 20 mM HCOOH and 5 % acetonitrile**
Mobile phase B	**5 % 20 mM HCOOH and 95 % acetonitrile**
Detection wave length	**214 nm**
Flow rate	**0,3 ml/min**
Gradient	**0 minute – 25 % mobile phase B 14 minutes – 60 % mobile phase B 15 minutes – 100 % mobile phase B 17 minutes – 100 % mobile phase B 17,5 minutes – 25 % mobile phase B 24 minutes – 25 % mobile phase B**
Column temperature	**23°C**
Injection volume	**15 µl**
Analysis time	**24 minutes**

3.5 Stock solutions

Stock solutions containing 1 mg/ml of each of the drug analytes are prepared as shown in Tab. 5. All of the solutions are protected from light except from stock solution 1 which is also stored at 4°C. Stock solution 5 is placed in ultrasonic bath for 15 minutes in order to dissolve all the drug analytes.

Table 5 Stock solutions

	Drug analytes	Dissolved in
Stock sol. 1	**Pethidine, nortriptyline methadone, haloperidol and loperamide**	**ethanol**
Stock sol. 2	**Cimetidine, metoprolol, hydralazine**	**ethanol**
Stock sol. 3	**Pindolol, salbutamol, metaraminol, mianserine**	**ethanol**
Stock sol. 4	**Procaine, hydroxyzine, papaverine, promethazine, fluphenazine, clomipramine, verapamil**	**ethanol**
Stock sol. 5	**Perphenazine, pyrilamine, clozapine, prochlorperazine, reserpine**	**1 M DMSO in ethanol**

3.6 Standard solutions

Standard solutions at 10 µg/ml are prepared weekly by diluting the stock solutions with 10 mM HCl. All of the solutions were stored at room temperature and protected from light.

3.7 Donor solutions

Donor solutions containing spiked aqueous samples at 1 µg/ml are prepared daily by diluting the standard solutions with 10 mM HCl. Different concentrations of NaCl-solution was added to the donor solutions as described in Tab. 6.

3.8 Acceptor solutions

8,357 ml 37 % HCl was diluted with deionized water (MilliQ) to a total volume of 100 ml and a final concentration of 1 M in a volumetric flask. Solutions with lower molarities are prepared by dilution from this solution.

3.9 Organic solvents

5 % DEHP in NPOE

43,6 mg of DEHP was added to 0,83 g of NPOE

10 % DEHP in NPOE

154,4 mg of DEHP was added to 1,39 g of NPOE

25 % DEHP in NPOE

343,0 mg of DEHP was added to 1,03 g of NPOE

25 % DEHP in ENB

280,0 mg of DEHP was added to 0,84 g of ENB

25 % TEHP in ENB

243,0 mg of TEHP was added to 0,73 g of ENB

The major parts of the experiments were performed with either pure ENB or NPOE as organic solvent to impregnate the porous hollow fiber.

3.10 NaCl solutions

Two methods were used in preparing NaCl-solutions; the first one was by weighting different amount of the solid matter directly into the 10 ml volumetric flask and dissolved it to volume in donor solutions. The other method was making a 20% (w/v) NaCl-solution in deionized water, and for each of the required NaCl concentration, different amounts of the 20% (w/v) NaCl- solution was added into the 10 ml volumetric flask. Preparation of different NaCl concentration utilizing the latter method was further illustrated in Tab. 6.

Table 6 Schematic preparation of different NaCl concentrations

Volume	Final concentration of drug analytes in the donor solution	Desired NaCl concentration % (w/v)	Added in 10 ml volumetric flask		
			Standard solution 10 µg/ml	**20 % NaCl-solution**	**10 mM HCl**
10 ml	**1 µg/ml**	**10 %**	**1 ml**	**5 ml**	**Added to the calibration mark**
10 ml	**1 µg/ml**	**7,5 %**	**1 ml**	**3,75 ml**	
10 ml	**1 µg/ml**	**5 %**	**1 ml**	**2,5 ml**	
10 ml	**1 µg/ml**	**2,5 %**	**1 ml**	**1,25 ml**	
10 ml	**1 µg/ml**	**1 %**	**1 ml**	**0,5 ml**	
10 ml	**1 µg/ml**	**0 %**	**1 ml**	**0 ml**	

3.11 Separation buffer for CE

Phosphate buffer pH 2,7

1,725 g NaH_2PO_4 was dissolved in deionized water (MilliQ) to a volume of 500 ml 25 mM NaH_2PO_4 solution. 169 µl orto-phosphoric acid 85 % was diluted with distilled water (MilliQ) to a volume of 100 ml. 25 mM orto-phosphoric acid was titrated to 25 mM NaH_2PO_4 solution with a pH meter to get a pH of 2,7. The buffer was finally filtrated through a 0,45 µm Minisart RC 25 single use syringe filter to remove particles.

3.12 Mobile phases for HPLC

Mobile phase A consisted of 95 % 20 mM HCOOH and 5 % acetonitrile. 20 mM HCOOH solution was prepared by diluting 755 µl of HCOOH with deionized water (MilliQ) to 1l in a volumetric flask. 50 ml acetonitrile was added to 450 ml of 20 mM HCCOH to make the mobile phase A. In preparation of mobile phase B 50 ml of 20 mM HCOOH was added to 450 ml acetonitrile. The bottles were finally placed in ultrasonic bath for 30 minutes to remove the air bubbles.

3.13 Other solutions

0,4 g NaOH was dissolved in a 100 ml volumetric flask with deionized water (MilliQ) to a prepare a concentration of 100 mM NaOH solution.

4 g NaCl was dissolved in a 20 ml volumetric flask with deionized water (MilliQ) to prepare 20 % (w/v) NaCl- solution.

6 mM imidazole with 4 mM formic acid solution was used for indirect UV-detection of Na^+ ions, while 5 mM Chromate with 0,5 mM TTAB solution was used for indirect UV-detection of Cl^-. Internal standard of KCl and NaCl was used in both experiments. The solutions were borrowed from a lab colleague.

3.14 Calculation of recovery and enrichment

The recoveries from EME were calculated utilizing the following equation for each drug analyte [1]:

$$recovery(\%) = \frac{n_a}{n_s}\ x\ 100\ \% = \frac{v_a}{v_s}\frac{c_a}{c_s}\ x\ 100\ \%$$

(3-1)

where n_a and n_s represented the number of moles in the acceptor solution (after the extraction) and the donor solution (before the extraction), respectively. V_a and C_a are the volume and concentration of the acceptor solution after extraction. V_s and C_s are the volume and the concentration of the donor solution before the extraction. Enrichment (E) during EME is calculated utilizing the following equation for each drug analyte [7]:

$$E = \frac{C_a}{C_s}$$

(3-2)

3.15 Statistics

Analytical results in this work were reported with mean value, standard deviation and relative standard deviation to ensure the scientific quality the concluding remarks will be based upon. The relative standard deviation (RSD) must be small and constant in order to achieve high precision. High precision indicated good reproducibility of the method and desirable in the field of analytical research. The RSD value was calculated according to the following equation [2]:

$$RSD\ (\%) = \frac{s}{\bar{x}}\ x\ 100\ \%$$

(3-3)

where s represented the standard deviation and $\bar{x}$ described the mean value of the measurements.

3.15.1 T-test

In some cases it was necessary to perform a T-test to determine whether or not two measurements were significantly different regarding to the content of an analyte. Equations 3-4 and 3-5 were used to calculate the T-test:

$$S_{pooled} = \sqrt{\frac{s_1^2(n_1 - 1) + s_1^2(n_2 - 1)}{n_1 + n_2 - 2}} \tag{3-4}$$

$$t_{calculated} = \frac{|\bar{x}_1 - \bar{x}_2|}{S_{pooled}} \sqrt{\frac{n_1 n_2}{n_1 - n_2}} \tag{3-5}$$

Firstly the pooled standard deviation (S_{pooled}) for both of the measurements series was calculated, where s_1 and s_2 represented the standard deviation for the two series of measurements, and n_1 and n_2 corresponded to the number of measurements in series 1 and series 2 respectively (cf. equation 3-4). Thereafter, a value for $t_{calculated}$ (cf. equation 3-5) was compared to a corresponding t value table, typically at the 95 % confidence level. $\bar{x}_1$ and $\bar{x}_2$ represented the calculated mean value of each of the two measurements series. n_{1+} $n_2 - 2$ referred to the number of degrees of freedom. If the $t_{calculated}$ was higher than the value found in table for the chosen confidence level, then it was with x-% probability (typically 95 %) that the true values for the two measurements series was significantly different [2].

3.15.2 Q-test

If there was a single different measurement (outlier) which could not be removed from the data material, then a Q-test was performed before rejecting that outlier. The Q-test was calculated according to the following equation:

$$Q_{calculated} = \left|\frac{x_i - x_{critical}}{x_1 - x_{critical}}\right| \tag{3-6}$$

If the largest value was requested to be removed, the individual measurements must be ranged in an increasing order, starting from x_1 which represented the smallest value, x_i as the second largest value and $x_{critical}$ as the largest value. If the smallest value was desired to be rejected, then x_1 represented the largest value, x_i as the second largest value and $x_{critical}$ as the smallest value. Subsequently, $Q_{calculated}$ was compared to a corresponding Q-value table, and the outlier can be discarded if $Q_{calculated}$ was larger than the table value for a chosen confidence level [2].

4 Results and discussion

In this work, EME of basic drugs of different polarity was performed from samples with relative high content of NaCl ≤ 10% (w/v). The influence of NaCl on the extraction efficiency during EME has never been studied in detail before. Consequently, experiments had mainly been focused on optimizing various EME parameters in order to obtain reliable data material with respect to stable RSD values and high recovery. Twenty four basic drugs with log P in the range of -0,3 to 4,9 were extracted, and subsequently analysed in CE-UV. A few experiments were carried out with selected basic drug analytes to shed some light on whether the extraction kinetics (extraction time and applied voltage) was affected by the presence of NaCl concentration in the donor solution, and the analysis were performed in CE-UV and HPLC-UV for comparison purpose.

4.1 Recovery dependent on different NaCl concentrations

All the extractions for this experiment were carried out by extraction from 500µl aqueous donor solution spiked with 1µg/ml of each of the target analyte. The impregnated hollow fiber was filled with 15 µl 10 mM HCl as acceptor solution. The agitation speed was 900 rpm and the extraction time was 5 minutes.

4.1.1 Experiments with hydrophilic, basic drug analytes

Metaraminol, pindolol, metoprolol, mianserine, salbutamol, hydralazine, cimetidine were selected as they possessed a hydrophilic character with log P values in the range -0,3-1,6 and pK_a values in the range 2,3-9,8. Additionally, the electropherograms obtained from standard solutions of each of these drug analytes showed stable migration times and separated peaks. Based on earlier knowledge with hydrophilic drugs; the most suitable SLM, consisted of NPOE and 25 % DEHP, was used as well as an electrical potential of 50 V. DEHP was known as an efficient ion-pair reagent which can facilitate the transport of analytes across the SLM [11].

As summarized in Tab. 7, the recoveries for mianserine and metoprolol slightly decreased with increasing NaCl concentration. It was not clear whether the NaCl concentration or the physicochemical properties of the remaining hydrophilic drugs analytes affected the permeation at the interface between donor solution and the SLM, resulting in poor extraction recovery.

Table 7 Recovery and RSD with different NaCl concentrations in the donor solution.
SLM= NPOE + 25 % DEHP, electrical potential: 50 V, n = 3 replicates, nd = not detected, instrument= CE-UV

Drug analytes	Recovery (%)^n (RSD(%)^n)		
	0 % NaCl	5 % NaCl	10 % NaCl
Metaraminol	nd	-	nd
Pindolol	nd	-	4 (24)
Metoprolol	8 (17)	6 (26)	nd
Mianserine	6 (35)	-	2 (20)
Salbutamol	3 (42)	-	nd
Hydralazine	27 (24)	nd	nd
Cimetidine	10 (34)	-	nd

The next experiment was performed without NaCl in the donor solution, in order to study the recoveries as function of the applied electrical potential difference. From pure aqueous donor solutions spiked with 1 µg/ml of metaraminol, mianserine, pindolol and salbutamol, the drug analytes were extracted with an electrical potential of 100 V and 300 V. As described in Eq. (2-2) from section 2.1.4, the potential difference affected the dimensionless driving force of the mass transfer of analytes across the SLM. However, increased voltage did not give significantly higher recovery and lower the RSD values as seen from Tab. 8, bubble and gas formation were observed at the electrodes with 300 V.

Table 8 Recovery and RSD from EME with 100 V and 300 V
SLM= NPOE + 25 % DEHP, n = 3 replicates, nd = not detected, instrument=CE-UV

Drug analytes	Recovery (%)^n (RSD(%)^n)	
	100 V	300 V
Metaraminol	nd	nd
Mianserine	8 (18)	nd
Salbutamol	6 (45)	9 (38)
Pindolol	2 (35)	4 (33)

Interestingly, the potential difference across the SLM was not the limiting factor for the mass transfer of the actual analytes, and it was not an efficient way to circumvent the poor permeation at the interface between donor solution and the SLM. In the next experiment, extraction time was extended from 5 minutes to 10 minutes, and subsequent analyses were also performed in the donor solutions after each extraction to take a look into how the transport of analytes progressed. However, the electropherograms showed signs of co-migration which made it difficult to interpret and accordingly left out of result and discussion here.

4.1.2 Experiments with hydrophobic, basic drug analytes

Pethidine, nortriptyline, methadone, haloperidol and loperamide were selected in continuation of this work based on earlier experience and knowledge about their favourable physical-chemical properties regarding extraction in EME [7, 9]. Their pK_a- and log P values were shown in Tab. 1 in section 3.1. They were hydrophobic drugs with log P above 2 and pK_a values in the range 8,1-10,5. Fig. 3 showed the extraction recoveries obtained with different NaCl concentrations in the donor solution. A T-test was performed and it confirmed that the recovery for each drug analyte did not significantly differ under 0, 1, 5 or 10 % NaCl with 95 % confidence.

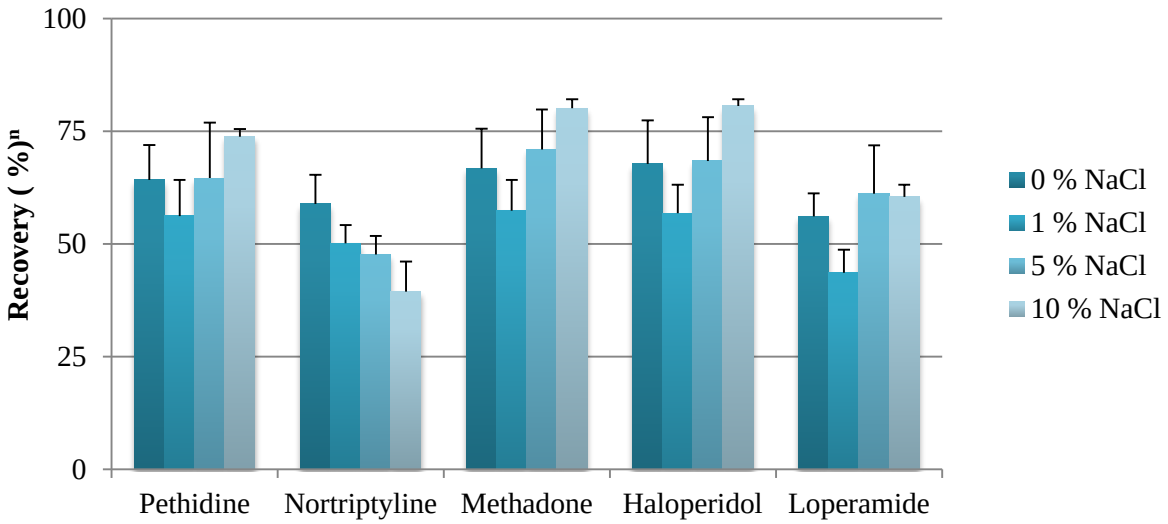

Figure 3 Recovery with different NaCl concentrations in the donor solution.
SLM= ENB, electrical potential: 25V, n = 3 replicates, RSD< 20 %, instrument = CE-UV

The same outcome was confirmed with the next experiment performed with 0% and 10% NaCl concentration in the donor solution. Recovery of pethidine, nortriptyline, methadone, haloperidol and loperamide was not dependent of the NaCl concentration. There was no explanation of this finding at this point, thus the next set of experiment was carried out with hydrophilic drugs in order to investigate if the result demonstrated the same tendency with NaCl.

Attention was directed to EME of several hydrophobic drugs in the next experiment in order to confirm that addition of NaCl did not affect the extraction recovery of hydrophobic drugs. Seven hydrophobic drugs analytes with log P above 2 and pKa values in the range 2,7- 9,7 was extracted with 0 % and 10 % NaCl in the donor solution, and the recoveries were illustrated in Fig. 4. 1 % and 5 % NaCl were excluded as they did not provide more information compared to 0 % and 10 % NaCl.

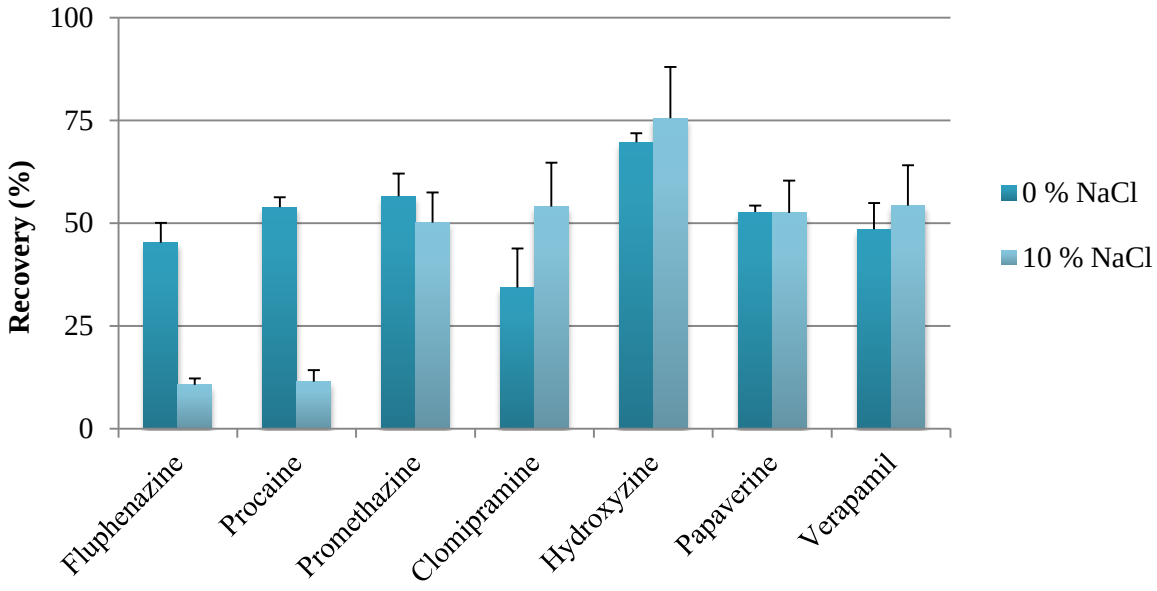

Figure 4 Recovery with different NaCl concentrations in the donor solution.
SLM= ENB, electrical potential =25 V, n = 3 replicates, instrument = CE-UV

A T-test was performed and it confirmed that there was a significant decrease in recovery for fluphenazine and procaine with addition of 10 % NaCl in the donor solution, and for the remaining five hydrophobic drugs, the NaCl concentration did not significantly influence the recovery with 95 % confidence. The RSD values for clomipramine and procaine was 27 % and 24 % respectively, which was higher than the RSD values below 20 % for the remaining extracted hydrophobic drugs. One explanation for the decrease in the recoveries of

fluphenazine and procaine was that both of these drug analytes had two positive charges in the acidified donor solution which might result in poor distribution into the SLM. Hydroxyzine was also a doubly charged analyte (pKa 2,1and 7,8), but the pH –value in the donor solution was below 2, thus only one of the basic functional group of the analyte was protonated.

In the next experiment, five basic drug analytes with two positive charges were extracted to study the effect of NaCl concentration on multiple charged analytes. They were hydrophobic with log P in the range 2,7-4,6 and pK_a values in the range 2,7-8,8. As seen in Fig. 5, the recoveries decrease significantly with increasing NaCl concentration confirmed with a T-test.

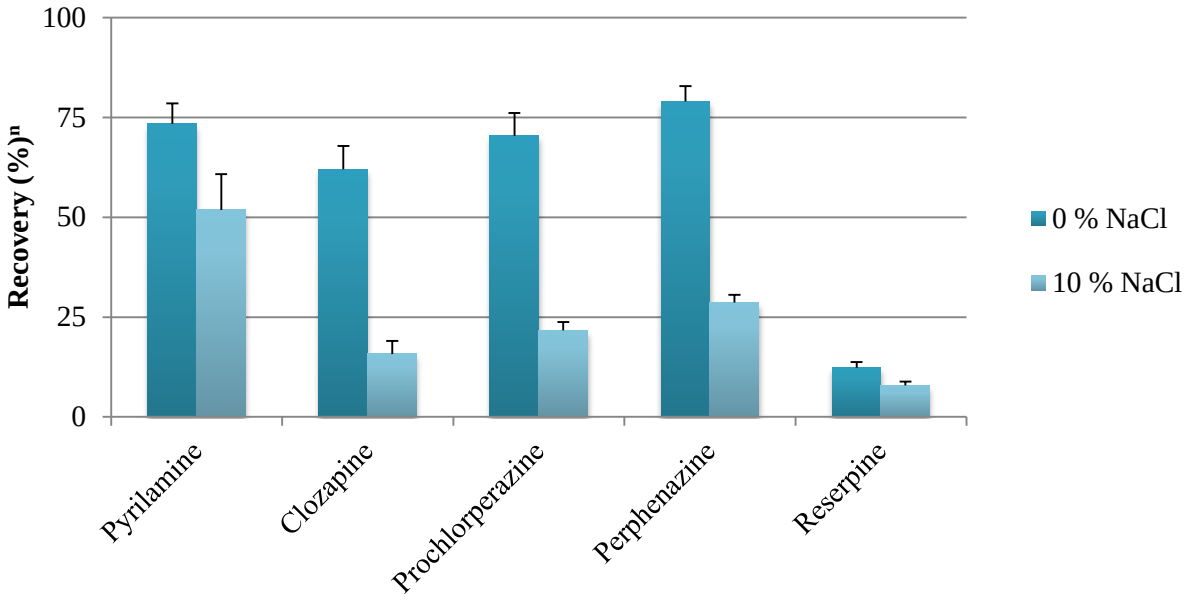

Figure 5 Recovery with different NaCl concentrations in the donor solution.
SLM= ENB electrical potential= 25 V, n = 6 replicates, RSD < 20 %, instrument= CE-UV

Obviously, the NaCl concentration affected the recovery of the actual analytes. However, this finding needed support from more data and a subsequent experiment with higher voltage and NPOE as the SLM was conducted, and the results were illustrated in Fig. 6.

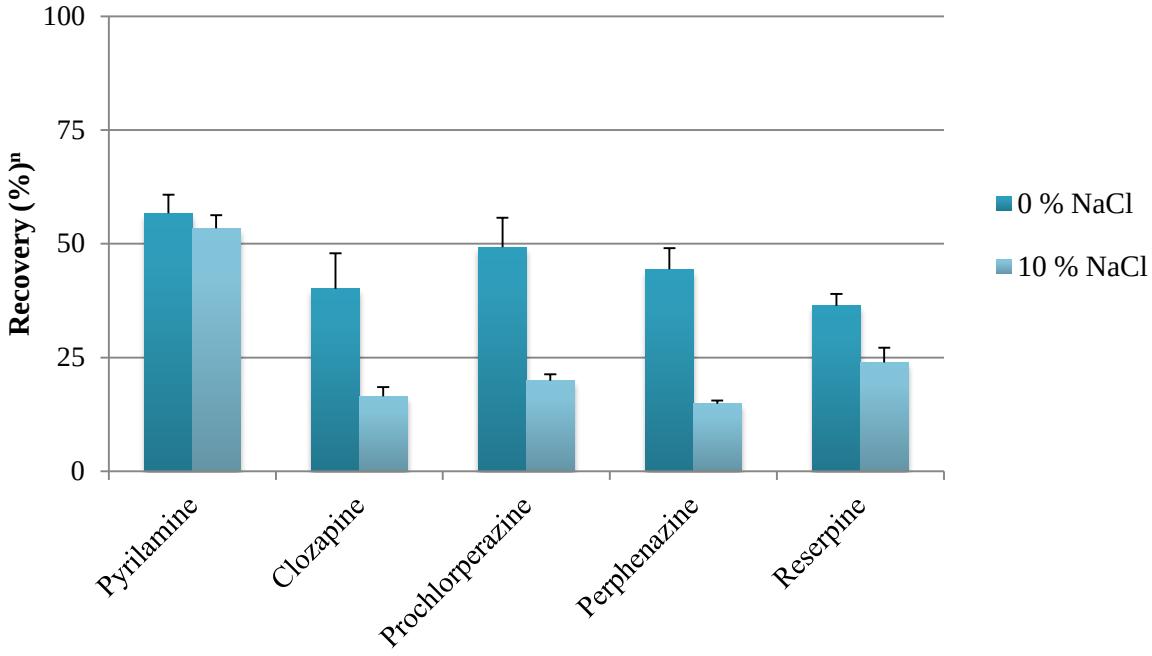

Figure 6 Recovery with different NaCl concentrations in the donor solution.
SLM= NPOE, electrical potential=300 V, n = 3 replicates, RSD < 20 %, instrument= CE-UV

T-test was performed and it confirmed earlier discoveries that 10 % NaCl in the donor solution significantly decrease the recovery of the actual analytes except for pyrilamine. Interestingly, increasing the electrical potential to 300 V and changing the chemical composition of the SLM does not give higher recovery compared to 25 V with ENB as the SLM. Thus, the following experiments were performed with ENB as the SLM, 25 V as the electrical potential.

4.1.3 Experiments with extraction times

The next experiment was performed with the same drug analytes to study the recoveries as function of extraction time. The intention was to collect more data on whether increased EME time would circumvent the influence of NaCl concentration on the doubly charged hydrophobic drugs. The results from 0 % NaCl in donor solution were summarized in Fig. 7, whereas the recoveries from 10 % NaCl in the donor solution were illustrated in Fig. 8.

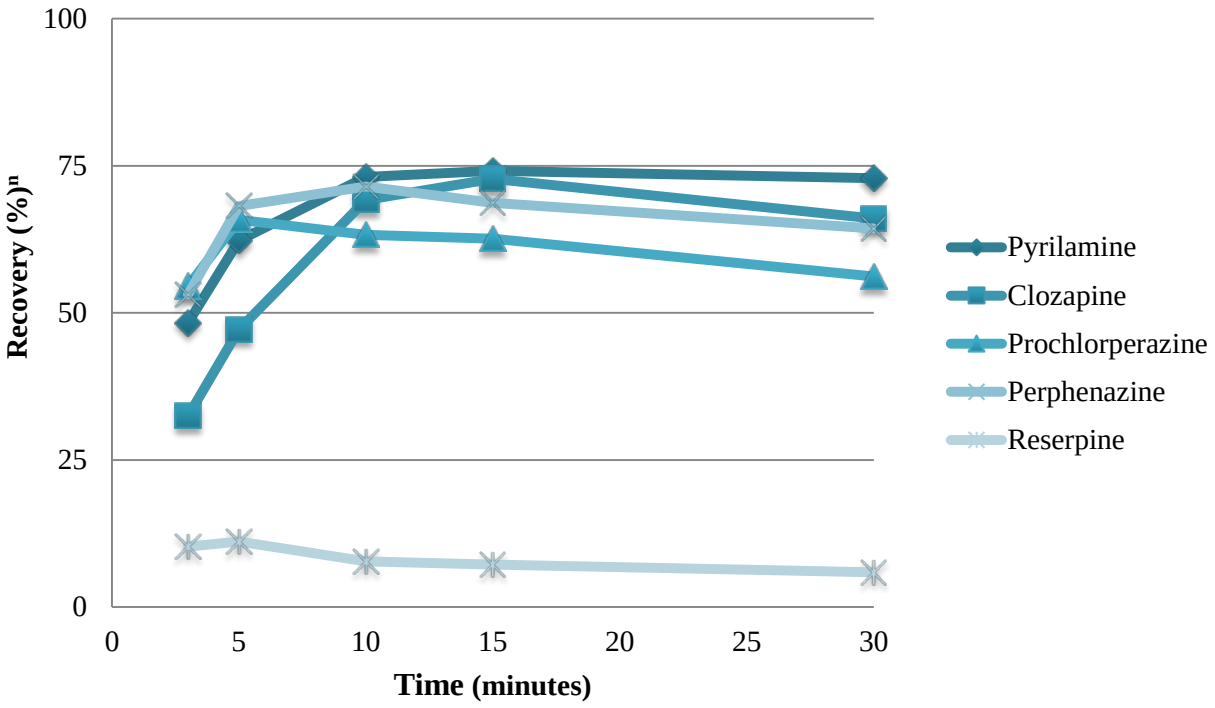

Figure 7 Recovery versus EME time with 0 % NaCl.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, n = 3 replicates, instrument= CE-UV

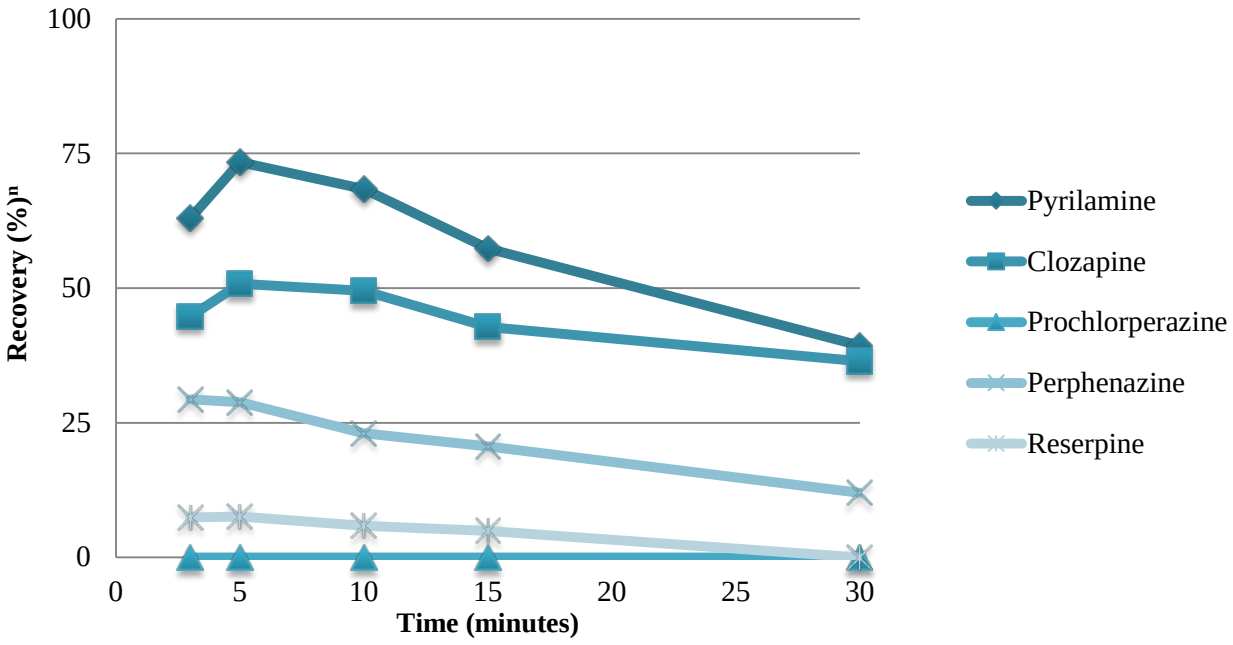

Figure 8 Recovery versus EME time with 10 % NaCl.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, n = 3 replicates, instrument = CE-UV

In Fig. 7, the recovery for each of the analyte corresponded to earlier reported work that when the EME system reached a steady-state between 5-10 minutes, no further gain in recovery of the target analytes was obtained [8, 10]. The highest recoveries with the smallest calculated RSD below 15 % were obtained after 10 minutes of extraction time for pyrilamine, clozapine, prochlorperazine and perphenazine. The low recovery of reserpine did not increase with increasing time, and one explanation could be that it was the largest in terms of molecule weight with highly bulky properties causing poor electrophoretic mobility within the SLM, or at the interface between the donor solution and the SLM [11]. Fig. 7 also illustrated experimentally that different analytes required different extraction time to reach steady-state as pointed out from earlier work [8, 10].

However, in Fig. 8, only the data obtained for pyrilamine and clozapine from 3 minutes of extraction with NaCl was reliable and reproducible, with RSD values below 5 %. Even though the recoveries was highest after 5 minutes of extraction, the RSD values for pyrilamine and clozapine was 24 % and 12 % respectively. After 10 and 15 minutes of EME, the recoveries of pyrilamine and clozapine decreased significantly. Around 30 minutes, the RSD value rose to 40 %. Prochlorperazine was not detected at any given extraction time with 10 % NaCl in the donor solution as illustrated in Fig. 8. The recoveries obtained for perphenazine and reserpine was tied to high RSD values above 29 % at each level of extraction time. The reason behind the rise in RSD values was not clear, but the NaCl concentration might have affected the electrophoretic mobility of the actual analytes across the SLM with prolonged extraction time.

The next experiment was performed with the same drug analytes to study whether there was a linear relationship which described the fall in recovery from 0 % to 10 % NaCl in the donor solution. The results were summarized and illustrated in Fig. 9. As observed from earlier experiments with the same analytes, data obtained for prochlorperazine, perphenazine and reserpine were tied with instability characterized by high RSD values above 50 %. As for pyrilamine and clozapine there was clearly no linear relationship from 0 % to 10 % NaCl concentration in the donor solution.

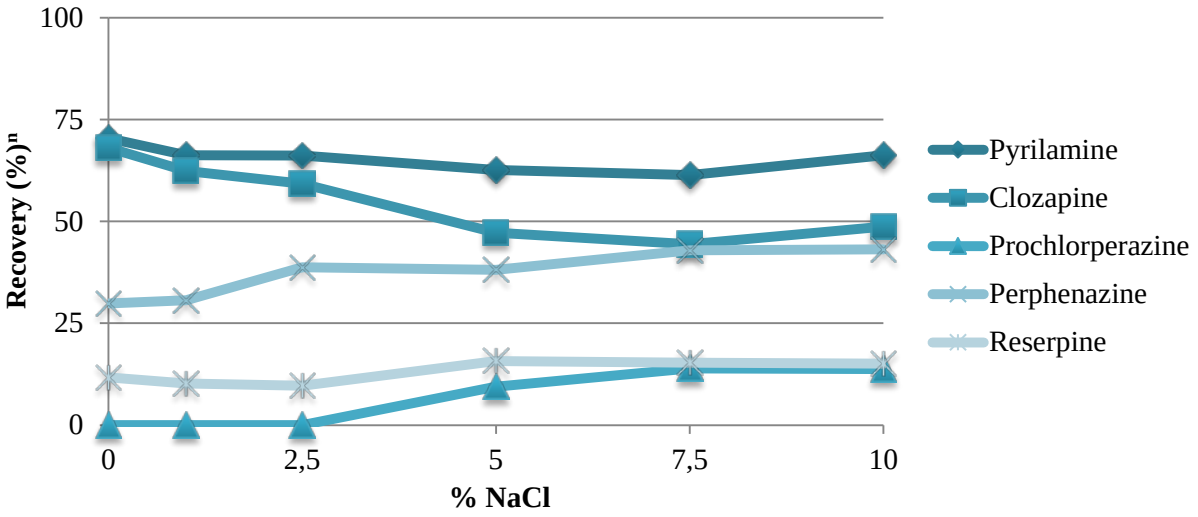

Figure 9 Recovery versus different NaCl concentrations.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, extraction time=5 minutes n = 3 replicates, instrument= CE-UV

The same experiment was performed twice to eliminate any operating errors and reduce the RSD values. However the outcome was still the same, very unreliable data obtained due to high calculated RSD values of the extraction recovery of prochlorperazine, perphenazine and reserpine.

To address the challenge with high RSD values, there was some discussion about the probability of salting-out effect when weighing out solid NaCl directly into the volumetric flask containing donor solutions. Salting-out effect was defined as reducing the analyte partitioning into the aqueous solution due to dissolved NaCl [25]. This could change the stability of the donor solutions containing different NaCl concentrations as the volumetric flask stood on the laboratory bench for several hours during the experiment. This was not a problem with the initially experiments. Thus, to avoid further problem, the following experiments were performed with 20 % (w/v) NaCl solution added to the donor solution in different volumes for each required NaCl concentration as described in Tab. 6 in section 3.10.

The next experiment was performed to confirm the influence of NaCl concentration on pyrilamine, clozapine, prochlorperazine, perphenazine and reserpine. The recoveries were summarized in Fig. 10. T-test was performed and it proved that there was a significant decrease in recovery of pyrilamine, clozapine and perphenazine with 10 % NaCl in the donor solution compared to 0 %. Prochlorperazine was not detected with NaCl and the recovery of reserpine was not altered by NaCl in the donor solution. However, this result was inconsistent with the result illustrated in Fig. 6. The reason behind this finding was not clear; the only difference between the two experiments was the preparation of the NaCl concentration in the donor solution.

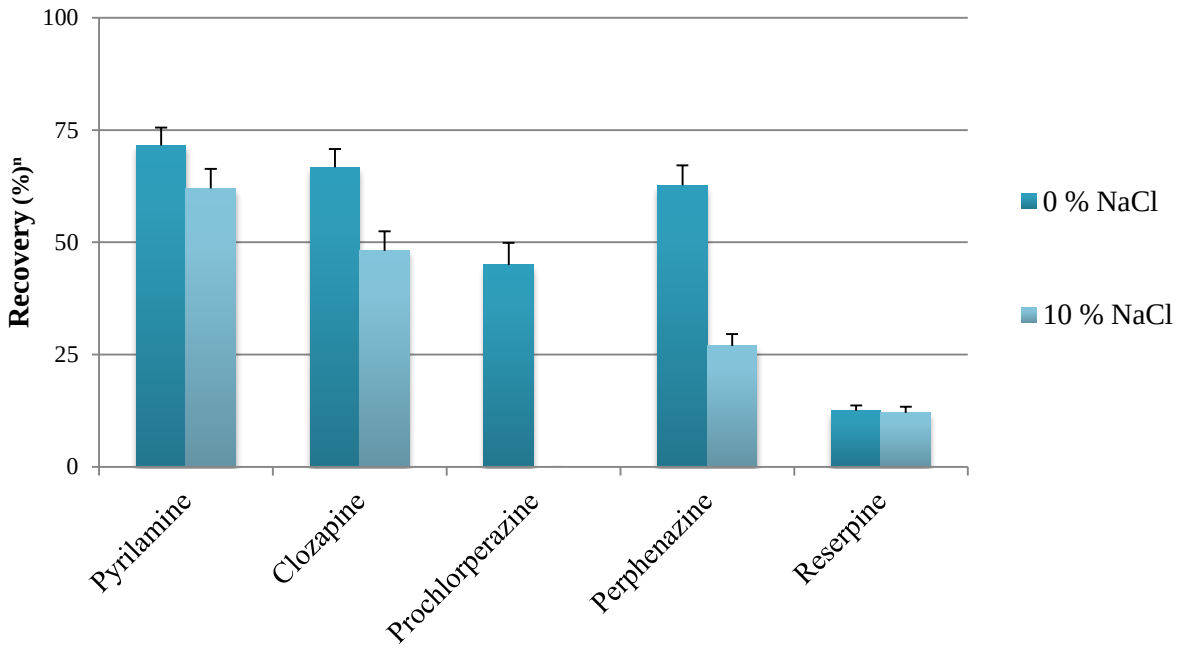

Figure 10 Recovery with different NaCl concentrations.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, extraction time=5 minutes n = 6 replicates, RSD < 16,1 %, instrument= CE-UV

The following experiment was performed with the same analytes to make sure that the results were supported with sufficient, reliable data. As illustrated in Fig. 11, the recoveries of clozapine and perphenazine were significantly decreased with 95 % confidence after calculation of a T-test. The NaCl concentrations did not affect the recovery of pyrilamine and reserpine with RSD values below 15 % for each level of NaCl concentration, while the RSD values for clozapine and perphenazine were below 22 % and 31 % respectively.

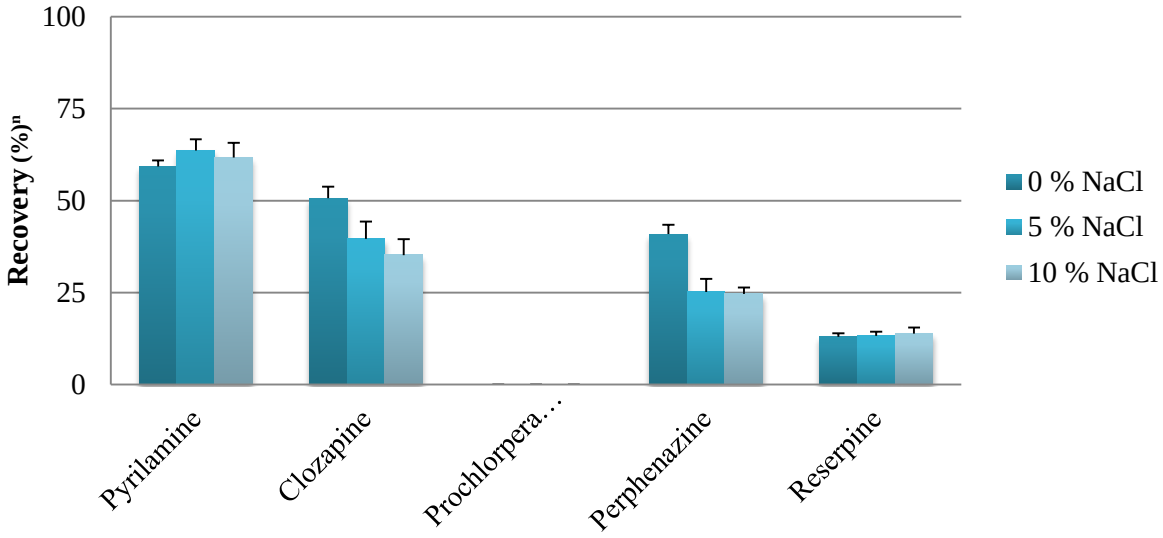

Figure 11 Recovery with different NaCl concentrations.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, extraction time=5 minutes n = 6 replicates, instrument= CE-UV

4.1.4 Experiments with different donor compartments

A different approach was made in order to reduce the RSD values and find an explanation behind the inconsistent results of pyrilamine, clozapine, prochlorperazine, perphenazine and reserpine with different NaCl concentrations. Three different formats of vials as shown in Fig. 12 were tested in addition to the previously used glass vial as donor compartment in EME.

Figure 12 Different format of vials utilized for EME. A = 1,5 ml glass vial, B= 1,5 ml polypropylene (PP) vial, C= 1,5 ml silanized vial, D= 2 ml microtube of polypropylene

Consequently, in the next experiment with pyrilamine, clozapine, prochlorperazine, perphenazine and reserpine, the former EME conditions were kept and only the previously used glass vial was changed with a microtube of polypropylene. The results were summarized in Fig. 13.

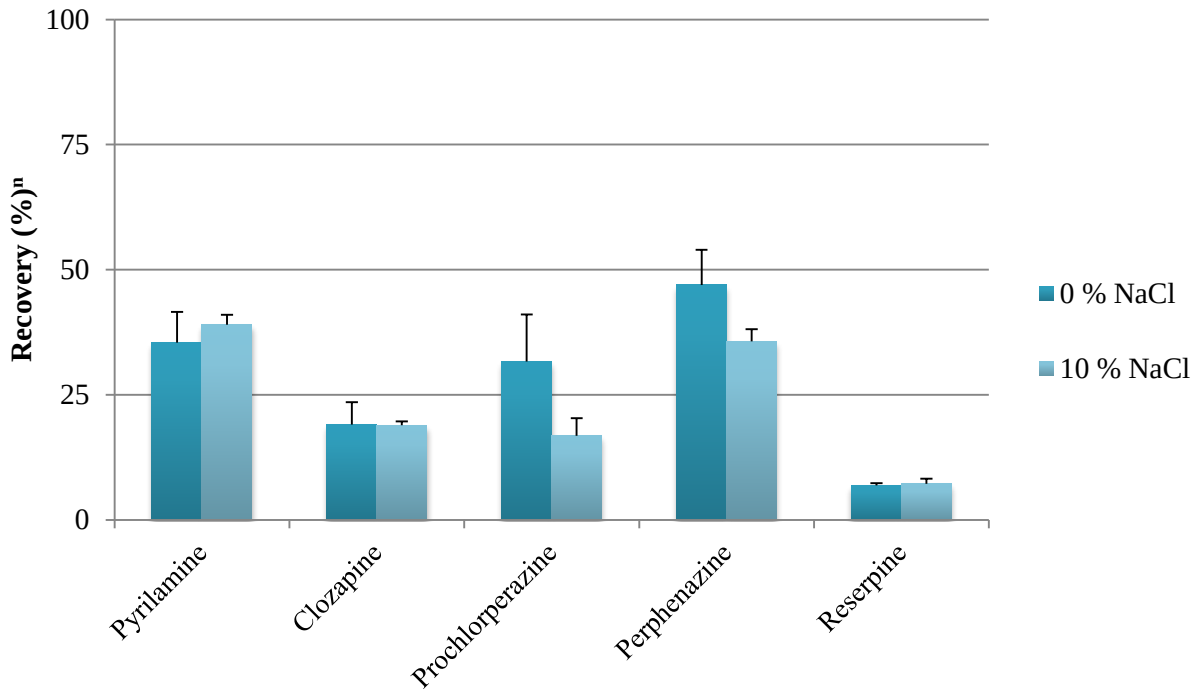

Figure 13 EME in a microtube with different NaCl concentration.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, extraction time=5 minutes n = 3 replicates, instrument= CE-UV

Data obtained for prochlorperazine was difficult to interpret as the RSD value was above 29 %. However, the RSD values of the extraction recoveries of the remaining analytes were below 15 % in 10 % NaCl. Thus, a T-test was carried out and it confirmed that there were no significant differences in the recoveries obtained from 0 % and 10 % NaCl in the donor solution. More information was important before making any conclusion about the nature of donor compartment could affect the recoveries of the actual analytes. In the following experiment the extraction time was extended from 5 minutes to 15 minutes to study whether it was possible to achieve higher recoveries in the microtube. The results were illustrated in Fig. 14.

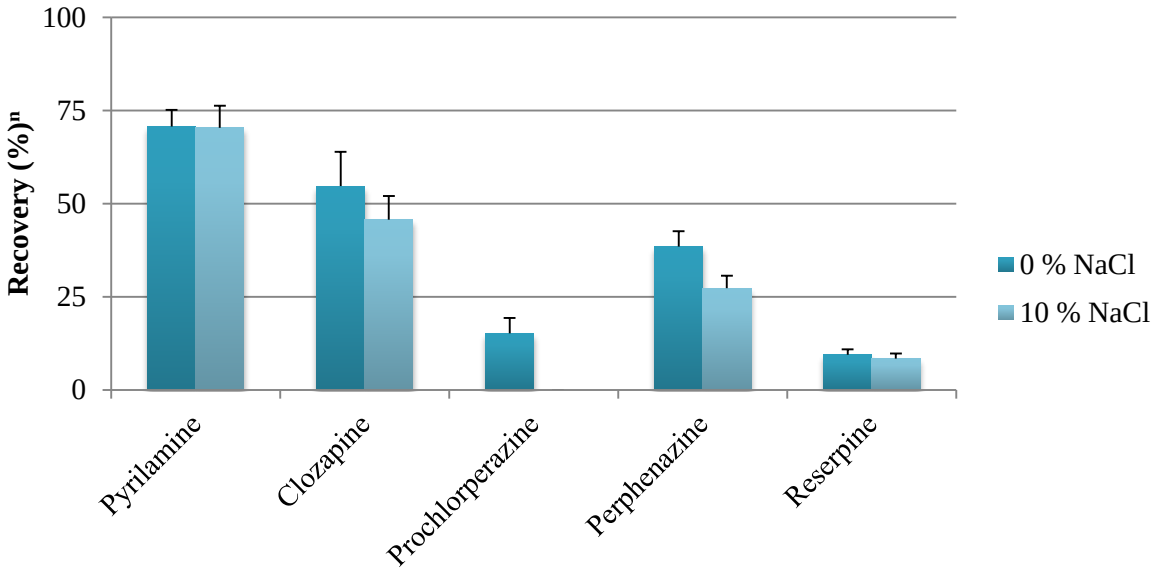

Figure 14 EME in a microtube with different NaCl concentration.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, extraction time=15 minutes, n = 6 replicates, instrument = CE-UV

The RSD value of the recoveries of prochlorperazine with 0 % NaCl was above 28 %, which made the analysis result unreliable and the drug analyte was not detected with 10 % NaCl. However, a T-test was performed for the remaining drug analytes which had RSD values below 16 %, and it proved that there was no significant decrease in the recoveries of pyrilamine, clozapine and reserpine with addition of 10% NaCl except for perphenazine. Increasing the extraction time in the microtube only increased the recoveries of pyrilamine and clozapine.

More experiments were required to address the challenge of different result outcome when changing from the glass vial to the polypropylene microtube as donor compartment during EME. The same drug analytes were extracted under the same EME conditions in both experiments, except from different donor compartment material. There was a possibility of salting-out effect in the glass vial compared to the polypropylene microtube. One laboratory test of absorption to glass vials was carried out to take a further look into this detail. However, the experiment demonstrated that the target analytes did not absorb to the surface of the glass vial in the presence of NaCl concentration.

The intention behind the following experiment was to study whether the surface of the material inside the donor compartments produced any electricity or held any charge effect after prolonged time of extraction. Hence, the next experiment was performed using glass, polypropylene (PP) and silanized vials of the same geometry as donor compartments. The drug analytes of interest were pethidine, nortriptyline, methadone, haloperidol and loperamide as the recoveries of these analytes were not dependent on the NaCl concentration which was of significant finding. The results were summarized in Tab. 9.

Table 9 Extraction time and different vials with 0 % NaCl.
SLM= ENB, electrical potential=25V, n=5 replicates, instrument = CE-UV

Drug analytes	Recovery (%)^n (RSD(%)^n)								
	5 minutes			10 minutes			20 minutes		
	Glass	PP	Silanized	Glass	PP	Silanized	Glass	PP	Silanized
Pethidine	74 (10)	74 (4)	87 (11)	83 (5)	91 (5)	94 (6)	86 (5)	94 (4)	107 (7)
Nortriptyline	76 (6)	80 (6)	92 (4)	78 (6)	81 (6)	87 (7)	80 (5)	82 (5)	97 (9)
Methadone	83 (6)	90 (3)	100 (3)	88 (4)	95 (6)	97 (5)	90 (4)	94 (3)	108 (7)
Haloperidol	77 (7)	86 (9)	93 (3)	80 (4)	89 (6)	91 (6)	82 (4)	88 (5)	101 (8)
Loperamide	58 (7)	68 (8)	76 (2)	60 (4)	69 (5)	70 (6)	61 (4)	70 (8)	80 (9)

As seen in Tab. 9, there was an increase in recovery of each of the target analytes after 10 minutes of extraction utilizing glass and polypropylene vials as donor compartment. Clearly, there was a difference in the recoveries obtained from EME in glass, polypropylene and silanized vials without NaCl concentration.

The next experiment was carried out with pethidine, nortriptyline, methadone, haloperidol and loperamide. The NaCl independence of the actual analytes was tested in glass, polypropylene and silanized vials as donor compartment after 5 minutes of EME. The results from analysis in HPLC-UV were summarized in Tab. 10, and interestingly, the RSD values calculated from the recoveries of each of the analytes with 10 % NaCl concentration was smaller compared to the absent of NaCl concentration in the donor solution.

Table 10 EME of monocharged analytes in 5 minutes.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, n = 6 replicates, instrument = HPLC-UV

Drug analytes	Recovery (%)^n (RSD(%)^n)					
	0 % NaCl			10 % NaCl		
	Glass	PP	Silanized	Glass	PP	Silanized
Pethidine	80 (26)	89 (12)	84 (7)	110 (8)	85 (8)	102 (9)
Nortriptyline	105 (16)	118 (9)	112 (9)	122 (7)	111 (7)	110 (6)
Methadone	94 (14)	93 (12)	86 (10)	35 (10)	32 (9)	33 (11)
Haloperidol	111 (12)	114 (9)	105 (10)	114 (7)	106 (4)	105 (6)
Loperamide	93 (7)	93 (11)	85 (13)	97 (9)	89 (8)	77 (7)

In the next experiment, attention was directed to the challenge of hydrophobic, doubly charged analytes. A tendency of decreased recoveries with increased NaCl concentration was observed from the experiments performed so far. However, the data obtained showed signs of inconsistent results connected with high RSD values. Further information was needed to study whether the high RSD was due to the relative high content of NaCl which was 10 % in the donor solution. Thus, the next experiment was performed with 0 % NaCl and 5 % NaCl in three different donor compartments, and the results were summarized in Tab. 11.

Table 11 EME of doubly charged analytes in 5 minutes.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, n = 6 replicates, instrument = CE-UV

Drug analytes	Recovery (%)^n (RSD(%)^n)					
	0 % NaCl			5 % NaCl		
	Glass	PP	Silanized	Glass	PP	Silanized
Pyrilamine	74 (14)	65 (7)	72 (9)	73 (3)	57 (18)	68 (20)
Clozapine	55 (17)	46 (13)	59 (12)	42 (16)	28 (26)	38 (39)
Prochlorperazine	70 (8)	62 (15)	70 (8)	44 (21)	43 (29)	39 (26)
Perphenazine	86 (9)	76 (8)	85 (5)	62 (11)	59 (18)	60 (15)
Reserpine	12 (11)	11 (10)	12 (13)	15 (24)	13 (38)	16 (15)

As seen in Tab. 11, the nature of the donor compartment did not circumvent the low recoveries of the actual analytes from donor solutions containg NaCl. With 5 % NaCl in the donor solution, the RSD values for clozapine and prochlorperazine was above 30 % in extractions from polypropylene and silanized vials. Results from extractions in glass vials were more reliable as the RSD value ranged from 11 – 24 %. The recoveries of pyrilamine and reserpine remained unaffected by the NaCl concentration in contrast to clozapine, prochlorperazine and perphenazine.

In the next experiment, the same drug analytes was extracted for 20 minutes in glass, polypropylene and silanized vials with 5 % NaCl in the donor solution. As summarized in Tab. 12, the results confirmed that the nature of the donor compartments did not alter the recoveries of each analytes in the presence of NaCl. The recovery of pyrilamine after 20 minutes of EME remained unaffected by the change in the material of the donor compartment with 5 % NaCl added. This finding was supported by low RSD value in the range 8 - 10 % which indicated repeatable and reproducible data.

Table 12 EME of doubly charged analytes in 20 minutes.
SLM= ENB, electrical potential =25 V, agitation= 900 rpm, n = 6 replicates, instrument = CE-UV

Drug analytes	**Recovery (%)^n (RSD(%)^n)**		
	5 % NaCl		
	Glass	PP	Silanized
Pyrilamine	84 (8)	88 (10)	85 (8)
Clozapine	66 (25)	75 (8)	62 (20)
Prochlorperazine	43 (29)	47 (42)	44 (39)
Perphenazine	60 (19)	66 (26)	60 (20)
Reserpine	7 (30)	7 (15)	5 (40)

However, the high RSD values seen in Tab.12 might be reduced by reducing the voltage from 25 V to 10 V. The results were illustrated in Fig. 15. The calculated RSD values of the recoveries were below 20 %, however reducing the voltage led to smaller recoveries and it did not improve the data of perphenazine.

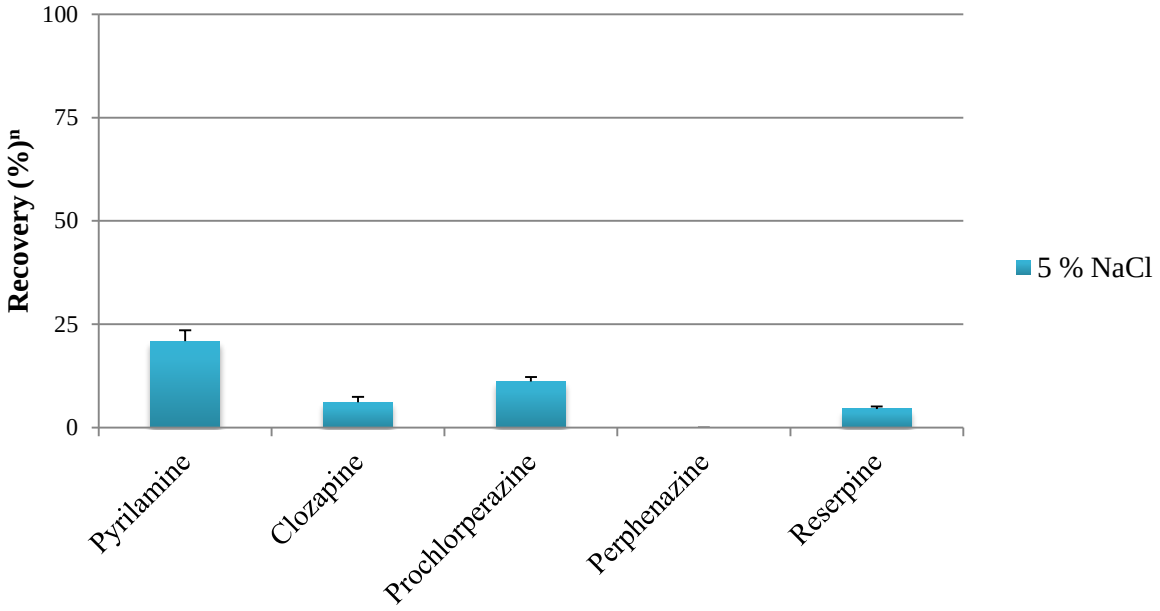

Figure 15. Recovery with 5 % NaCl concentration in the donor solution.
SLM= ENB, electrical potential =10 V, agitation= 900 rpm, extraction time=5 minutes, n = 6 replicates, instrument = CE-UV

4.2 Current dependent on NaCl concentrations

In order to investigate whether Na^+ ions in the donor solutions affected the current during EME, current measurements with pyrilamine, clozapine, prochlorperazine, perphenazine and reserpine as target analytes were performed in glass-, polypropylene- and silanized vials with and without 5 % NaCl. The results were illustrated in Fig. 16.

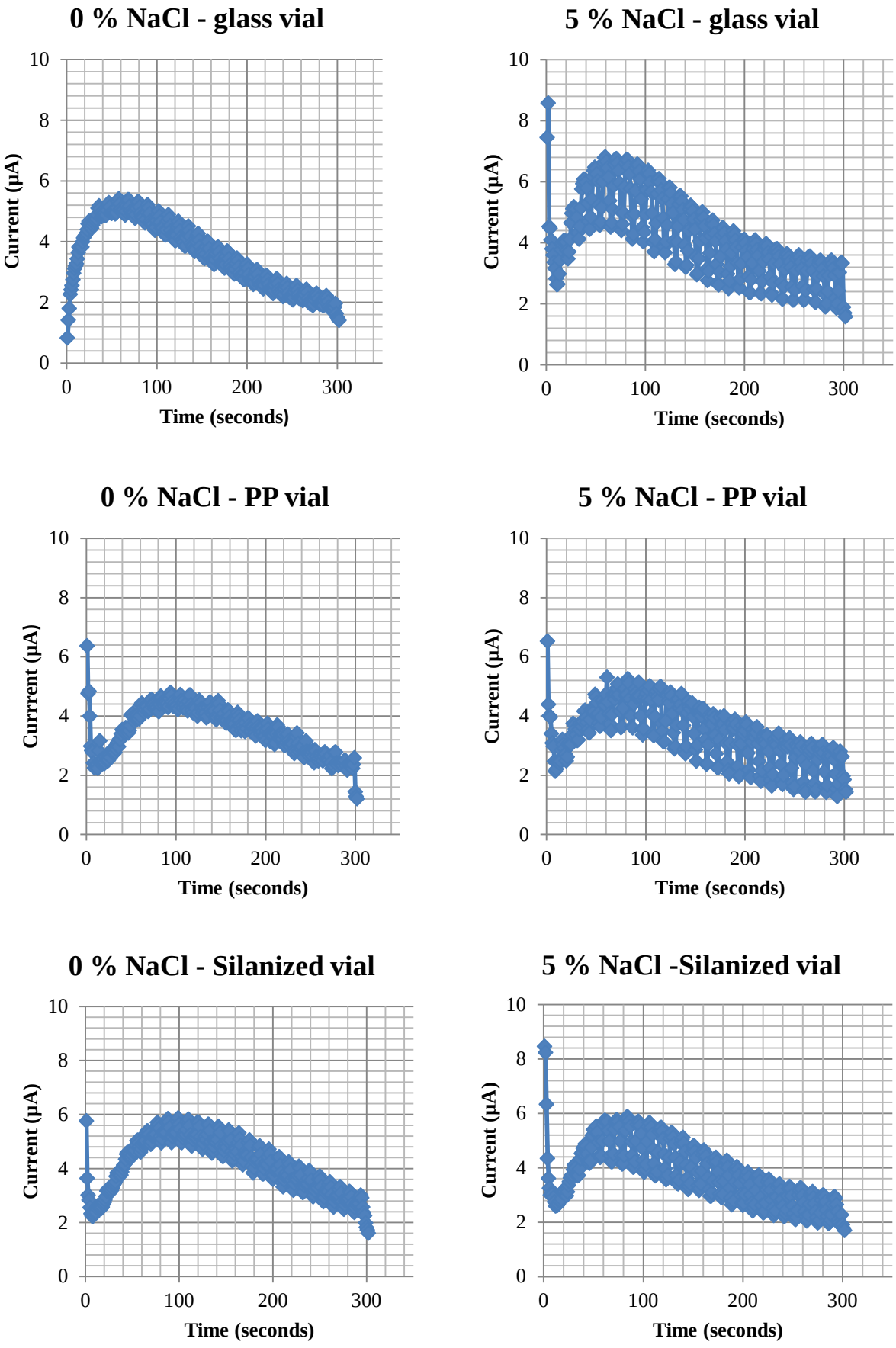

Figure 16 Current versus time with 0% and 5 % NaCl in the donor solution. SLM= ENB, Acceptor solution=15 µl 10 mM HCl, donor solution=500 µl spiked with 1µg/ml of each analyte, electrical potential =25 V, agitation= 900 rpm, extraction time=5 minutes, n = 6 replicates

As demonstrated in Fig. 16, the currents measurements showed signs of minor fluctuations of approximately 1 µA in the presence of 5% NaCl in the donor solutions after 5 minutes of EME time. However, the current level was very low and no electrolysis at the electrodes was observed. In general the current profile obtained from all three compartments correspond to earlier findings that electrical currents in the system peaked at the high initial concentration of charged analytes, decreased as the level of charged analytes in the donor solution was depleted and the system entered steady-state.

4.3 Detection of Na^+ in the acceptor solution

It was not clear if the minor fluctuations in the electrical currents flowing in the system were caused by cross membrane flux of Na^+ ions which in turn affected the electrokinectic migration of target analytes. This led to the next question about whether Na^+ ions were extracted into the acceptor solution. Thus, in the following experiment attention was directed to analysis of Na^+ in the acceptor solution which employed indirect UV-detection [26]. The principle behind indirect UV-detection was described in section 2.2.1. Imidazole and formic acid were used to make an electrolyte solution ("buffer"), which generated high background UV signal. When Na^+ ions passed through the detector, the background signal was reduced, producing a negative peak in the UV-signal, and the area of the peak produced was linearly related to the Na^+ ions concentration. The internal standard contained KCl and NaCl. Adjusting the separation conditions in CE was required and successful peaks (negative peaks in the UV-signal) for K^+ ions and Na^+ ions were obtained as shown in Fig.17.

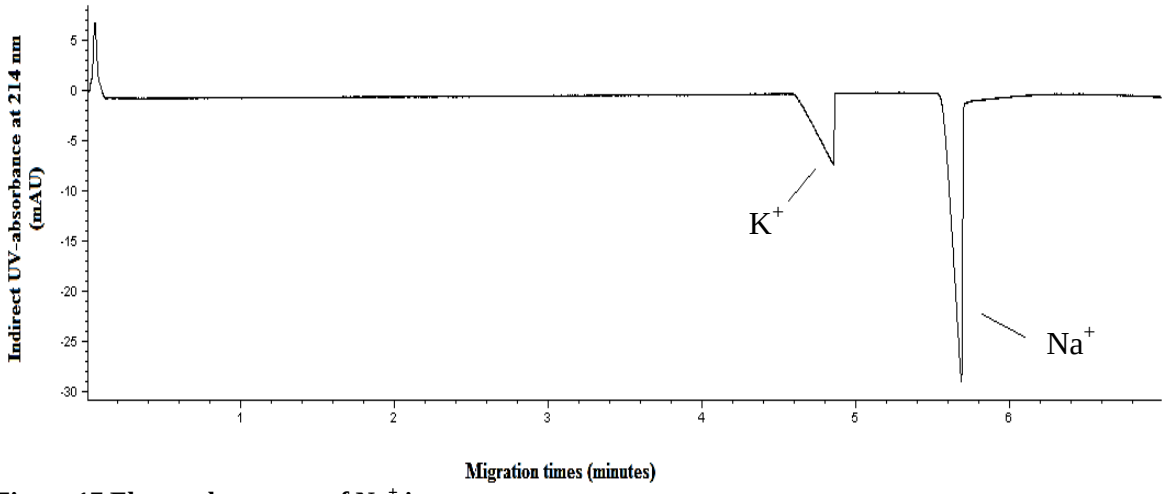

Figure 17 Electropherogram of Na^+ ions.

The results were summarized in Tab. 13, and it clearly demonstrated that Na^+ ions in the donor solution were not extracted across the SLM as peaks of Na^+ ions were not observed in the electropherogram obtained from the acceptor solution before and after EME with and without the NaCl concentration.

Table 13 Indirect UV-detection of Na^+ ions in acceptor and donor solution.
SLM= ENB, Acceptor solution=15 µl 10 mM HCl, electrical potential =25 V, agitation= 900 rpm, extraction time=5 minutes

		Na^+ ions peak in the electropherogram
I.	**Acceptor solution without EME**	Not detected
II.	**Acceptor solution after EME with 0 % NaCl in the donor solution**	Not detected
III.	**Acceptor solution after EME with 5 % NaCl in the donor solution**	Not detected
IV.	**Donor solution with 5 % NaCl after EME**	Detected
V.	**Deionized water without EME**	Not detected

4.4 The influence of Cl^- in the acceptor solution

Further experiments were performed to study whether Cl^- ions migrated from the acceptor solution toward the anode located in the donor solution. Cl^- was an inorganic anion which opposed the normal electroosmotic flow (EOF) in the CE-UV resulting in poor peak shapes and long analysis time [27]. Consequently, the EOF direction was reversed to detect Cl^- ions. Reversing the EOF direction required addition of a cationic surfactant which produced a bi-layer of positive charge on the capillary wall. Thus, the EOF and the anions migrated in the same direction promoted by a negative voltage in the CE-UV instrument [27]. Tetradecyltrimethylammonium bromide (TTAB) was used as the cationic surfactant and chromate was used as the background UV-absorbance for indirect detection of Cl^- in CE [27]. The results were summarized as peak areas in Tab. 14.

Table 14 Indirect UV-detection of Cl^- ions in acceptor and donor solution. SLM= ENB, electrical potential =25 V, agitation= 900 rpm, extraction time=5 minutes

	Peak area (mAU*s) of Cl
Before EME	
10 mM HCl	**180,3**
10 mM HCOOH	**11,9**
1 µg/ml of drug analytes in 10 mM HCOOH	**6,9**
H2O	**-**
After EME	
Acceptor solution 10 mM HCl	**121,2**
Donor solution: pure water	**-**
Acceptor solution 10 mM HCl	**92,9**
Donor solution: 10 mM HCOOH	**6,4**
Acceptor solution 10 mM HCl	**67,6**
Donor solution: 1 µg/ml of target analytes in 10 mM HCOOH	**4,9**

As shown in Tab. 14, Cl^- ions were detected in both the acceptor and donor solution before and after EME. However the amount of Cl^- ions was reduced in the acceptor solution after EME. One reason behind this was the possibility of production of $Cl_2(g)$ at the anode located in the donor solution.

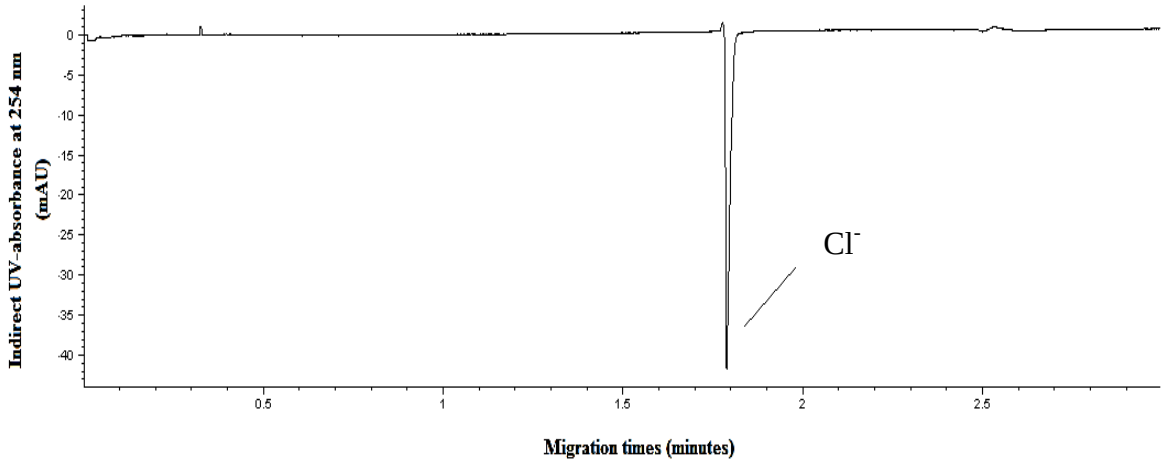

Figure 18 Electropherogram of Cl^- ions.

5 Conclusion

The present work has for the first time demonstrated that it is possible to perform EME of basic drugs of different polarity under relative high content of NaCl concentrations up to10 % (w/v). The recoveries did not depend on the NaCl concentrations in the donor solution for half of the drugs analytes tested, whereas the remaining drugs analytes demonstrated clearly poor distribution into the interface between the donor solution and the SLM in the presence of NaCl concentrations.

Different approaches were directed to this challenge with NaCl. Furthermore, a few parameters like applied voltage, extraction time, and the chemical composition of the SLM have been briefly investigated to maximize the recovery of the basic drugs and minimize the RSD values. However, more experiments must be conducted in order to fully understand how NaCl affected the distribution of basic drugs into the interface between the donor solution and the SLM.

For the first time, different materials of the donor compartment were tested for EME of basic drugs of different polarity. Silanized-, polypropylene- and glass vials of the same geometry were compared with respect to highest recovery achieved under different NaCl concentrations. The NaCl concentrations did not discriminate the nature of the donor compartment as the extraction recoveries were not altered by the change of the vial material.

6 References

1. Gjelstad, A. and S. Pedersen-Bjergaard, *Electromembrane extraction: a new technique for accelerating bioanalytical sample preparation.* Bioanalysis, 2011. **3**(7): p. 787-797.
2. Honoré Hansen, S., S. Pedersen-Bjergaard, and K.E. Rasmussen, *Introduction to Pharmaceutical Chemical Analysis*. Vol. First edition. 2012, United Kingdom: John Wiley & Sons Ltd.
3. Pedersen-Bjergaard, S. and K.E. Rasmussen, *Liquid-liquid-liquid microextraction for sample preparation of biological fluids prior to capillary electrophoresis.* Anal Chem, 1999. **71**(14): p. 2650-2656.
4. Petersen, N.J., et al., *Electromembrane extraction from biological fluids.* Anal Sci, 2011. **27**(10): p. 965-972.
5. Rasmussen, K.E. and S. Pedersen-Bjergaard, *Developments in hollow fibre-based, liquid-phase microextraction.* TrAC - Trends in Analytical Chemistry, 2004. **23**(1): p. 1-10.
6. Gjelstad, A., et al., *Kinetic aspects of hollow fiber liquid-phase microextraction and electromembrane extraction.* Anal Chim Acta, 2012. **742**: p. 10-16.
7. Pedersen-Bjergaard, S. and K.E. Rasmussen, *Electrokinetic migration across artificial liquid membranes. New concept for rapid sample preparation of biological fluids.* J Chromatogr A, 2006. **1109**(2): p. 183-190.
8. Gjelstad, A., et al., *Microextraction across supported liquid membranes forced by pH gradients and electrical fields.* J Chromatogr A, 2007. **1157**(1-2): p. 38-45.
9. Gjelstad, A., K.E. Rasmussen, and S. Pedersen-Bjergaard, *Simulation of flux during electro-membrane extraction based on the Nernst-Planck equation.* J Chromatogr A, 2007. **1174**(1-2): p. 104-111.
10. Middelthon-Bruer, T.M., et al., *Parameters affecting electro membrane extraction of basic drugs.* J Sep Sci, 2008. **31**(4): p. 753-759.
11. Gjelstad, A., K.E. Rasmussen, and S. Pedersen-Bjergaard, *Electrokinetic migration across artificial liquid membranes Tuning the membrane chemistry to different types of drug substances.* J Chromatogr A, 2006. **1124**(1-2): p. 29-34.
12. Dominguez, N.C., et al., *Selective electromembrane extraction at low voltages based on analyte polarity and charge.* J Chromatogr A, 2012. **1248**: p. 48-54.

13. Balchen, M., et al., *Electrokinetic migration of acidic drugs across a supported liquid membrane.* J Chromatogr A, 2007. **1152**(1-2): p. 220-225.
14. Balchen, M., L. Reubsaet, and S. Pedersen-Bjergaard, *Electromembrane extraction of peptides.* J Chromatogr A, 2008. **1194**(2): p. 143-149.
15. Balchen, M., et al., *Potential-driven peptide extractions across supported liquid membranes: investigation of principal operational parameters.* J Sep Sci, 2010. **33**(11): p. 1665-1672.
16. Gjelstad, A., K.E. Rasmussen, and S. Pedersen-Bjergaard, *Electromembrane extraction of basic drugs from untreated human plasma and whole blood under physiological pH conditions.* Anal Bioanal Chem, 2009. **393**(3): p. 921-928.
17. Eibak, L.E., et al., *Exhaustive electromembrane extraction of some basic drugs from human plasma followed by liquid chromatography-mass spectrometry.* J Pharm Biomed Anal, 2012. **57**: p. 33-38.
18. Jamt, R.E.G., et al., *Electromembrane extraction of stimulating drugs from undiluted whole blood.* Journal of Chromatography A, 2012. **1232**: p. 27-36.
19. Kjelsen, I.J., et al., *Low-voltage electromembrane extraction of basic drugs from biological samples.* J Chromatogr A, 2008. **1180**(1-2): p. 1-9.
20. Lodoen, C.P., et al., *Storage of oral fluid as dried spots on alginate and chitosan foam - a new concept for oral fluid collection.* Bioanalysis, 2013. **5**(3): p. 317-325.
21. Kuban, P., I.K. Kiplagat, and P. Bocek, *Electrokinetic injection across supported liquid membranes: new sample pretreatment technique for online coupling to capillary electrophoresis. Direct analysis of perchlorate in biological samples.* Electrophoresis, 2012. **33**(17): p. 2695-2702.
22. Pedersen-Bjergaard, S. and K.E. Rasmussen, *Extraction across supported liquid membranes by use of electrical fields.* Anal Bioanal Chem, 2007. **388**(3): p. 521-523.
23. Pedersen-Bjergaard, S. and K.E. Rasmussen, *Liquid-liquid-liquid microextraction for sample preparation of biological fluids prior to capillary electrophoresis.* Analytical Chemistry, 1999. **71**(14): p. 2650-2656.
24. Altria, K., A. Marsh, and C. Sanger-van de Griend, *Capillary electrophoresis for the analysis of small-molecule pharmaceuticals.* Electrophoresis, 2006. **27**(12): p. 2263-2282.

25. Endo, S., A. Pfennigsdorff, and K.U. Goss, *Salting-Out Effect in Aqueous NaCl Solutions: Trends with Size and Polarity of Solute Molecules.* Environmental Science & Technology, 2012. **46**(3): p. 1496-1503.
26. Altria, K.D., et al., *Inter-company cross validation exercise on capillary electrophoresis. Quantitative determination of drug counter-ion level.* Chromatographia, 1995. **40**(1-2): p. 47-50.
27. Altria, K.D., D.M. Goodall, and M.M. Rogan, *Quantitative determination of drug counter-ion stoichiometry by capillary electrophoresis.* Chromatographia, 1994. **38**(9-10): p. 637-642.